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NOTICE

This is to announce that in 1954 the name of this Journal (Canadian Journal of Medical Sciences) will be changed to ***Canadian Journal of Biochemistry and Physiology.*** This change of name is made to indicate that the scope of the Journal has been broadened to include nonmedical biochemistry. The present sequence of volume numbers will be retained and publication will continue to be on a bimonthly basis. The first number to be published under the new name will be issued in January 1954.

The subscription rate for this Journal effective 1 January 1954 will be $3.00.

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NUCLEASES IN THE CEREBROSPINAL FLUID

I. RIBONUCLEASE IN NORMAL, NEUROLOGICALLY NORMAL, AND IN PATHOLOGICAL CSF'S[1]

BY ERNEST KOVACS

Abstract

Ribonuclease (RNA-ase) was investigated in 800 individual CSF samples. The uranylacetate precipitation technique of MacFadyen was used, followed by total acid soluble phosphorus determinations (King) before and after incubation. Normal and neurologically normal CSF's showed low RNA-ase activity. The values ranged under 20% substrate depolymerized in 24 hr. A few exceptions showing high values were encountered: one specimen each of acute nasopharyngitis, 25%; infectious mononucleosis, 28%; and myositis, 30%. The meningitis CSF's showed a wide range of RNA-ase activity, 0–50%. Poliomyelitis specimens exhibited uniformly the highest values, 15–52%. This high RNA-ase activity paralleled chronologically the chromatolysis of the motoneurons and is of special significance. In the syphilis group only the neurosyphilis samples presented moderately high activity, but rarely attained the levels observed in meningitis and in poliomyelitis. In miscellaneous neurological conditions the highest activity was found in a specimen from a case of genuine epilepsy. The findings are discussed in detail.

Introduction

In the older literature uncertainty existed regarding the structure and decomposition of nucleic materials, (19, 10, 20, 11). Enzymic hydrolysis of highly polymerized nucleic acid starts with depolymerization of the macromolecules, brought about by the action of nucleases (15). Ribonucleic acid (RNA) is transformed into smaller molecular fragments by ribonuclease (RNA-ase) without liberating inorganic phosphorus, nitrogenous bases, or pentose (5, 6, 17, 18). Little exact work was done to demonstrate the presence and behavior of this enzyme in the CSF (25, 26). We have been interested in nucleic acid metabolism and have investigated a very large number of specimens for nuclease activity, using acceptable techniques. The result of RNA-ase assays forms the basis of the present publication.

[1] *Manuscript received June 30, 1953.*

Contribution from the Department of Hygiene and Preventive Medicine, University of Toronto, Ontario. This work was financed by a Mental Hygiene grant.

Methods

Substrate: Commercial yeast nucleic acid of Merck was purified by repeated precipitation with glacial acetic acid (17). In a few assays RNA of the Nutritional Biochemical Corporation was used, after similar purification. The different batches were alike in their physical and chemical properties and were assayed with standard enzyme preparation. Purified RNA solution (0.1%) was routinely used. Buffers: In most of the assays the RNA was dissolved in Michaelis' veronal–acetate buffer (24), of pH ± 7.2–7.36. In a small number of experiments, where spectrophotometrical measurements were made, the veronal buffer was not suitable, giving absorption bands in the ultraviolet regions under study. Borate–borax buffer of the same pH was used in these cases. Enzyme: 0.1 ml. of fresh or deep-frozen CSF was used as enzyme material. Cell free, centrifuged specimens were used in most of the assays (16). Activators: Magnesium sulphate and magnesium chloride were assayed in 0.01% final concentration. Preparation of the system for incubation: 0.1 ml. of CSF was added to 5.0 ml. of buffered substrate. The ingredients were mixed in glass-stoppered sterile bottles or plastic-capped large sterile test tubes and one drop of chloroform was added as preservative. Controls: In one control, saline was substituted for spinal fluid. In the second control, the substrate was replaced by buffer and activator in the required volume. Incubation: Incubation was carried out routinely for 24 hr. at 37° C., but higher and lower temperatures and varying time intervals were used occasionally to study the kinetics of the enzyme.

Determination of enzyme activity: Equal volumes of system and uranyl-acetate–trichloracetic acid reagent (MacFadyen (23)) were mixed and the test tubes incubated, after repeated shaking, for 15 min. at 30° C., to facilitate filtration (17). Acid-prepared phosphorus-free Whatman filter paper No. 42 was used throughout these assays. The total acid-soluble phosphorus was measured by King's method (14) in aliquots of the uranylacetate filtrate of the incubated system after perchloric acid digestion. The total phosphorus content of the system was also measured in aliquots (0.5–1.0) after perchloric acid digestion. Controls of CSF and the substrate separately were treated in the same way and their values deducted from the total acid soluble phosphorus values of the incubated mixture. By this method the effect of incubation on the acid soluble total phosphorus of the system was measured. The percentage of substrate hydrolyzed was calculated after determination of the total phosphorus content of the system.

The spontaneous hydrolysis of the substrate control was generally low but a small increase of total acid soluble phosphorus was always found (17). The phosphorus changes due to autolysis in buffered CSF have been described at length by us. If not controlled, they may represent a definite source of error (16). The nucleic acid content of the CSF specimens was measured colorimetrically and by ultraviolet spectrophotometer ((16) and unpublished observations). By our technique no appreciable amount of RNA was added to the substrate.

Results

Eight hundred individual CSF samples were assayed for RNA-ase activity. The results are illustrated in three tables (I-III) and one histogram (Fig. 1). In one hundred normal and neurologically normal specimens (Table I, Table II, Fig. 1), all but five are in the range 0 to 20% substrate depolymerized in 24 hr. at 37° C. The five exceptions are as follows: two neurologically normal specimens 21 and 22% respectively, nasopharyngitis 25%, infectious mononucleosis 28%, myositis 30%. Selected examples, including two exceptions, are shown in Table II, group I.

The over-all range in the meningitides group is from 0 to 50% (Table I, group B). The detailed values for the different etiological subgroups of the meningitides specimens indicate that the purulent meningitis samples (strepto- and staphylococcal) exhibited the highest change (50%), but that a large number of CSF remained within the normal ranges (up to 20%). The influenzal and pneumococcal subgroup is characterized by high activity; meningococcal meningitis specimens, like the purulent meningitis cases, show wide fluctuations. Table II, group II, presents selected illustrative meningitis samples. In repeated assays it was found that in about 10% of the specimens of this group RNA-ase activity was highest in the subacute stage, slightly lower in the acute, and definitely lower, sometimes nonexistent, in convalescence.

Values in the large poliomyelitis group ranged from 15 to 52%, as seen in Table I, group C, Table II, group III, and Fig. 1. In the nonparalytic form there were only four values under 20%; in paralytic poliomyelitis only two. In the bulbar form there were two and in polioencephalitis there were none under 20%. Table II, group III, shows selected examples. In the laboratory notes two numbers are given for each cell count: H = made at the bedside in hospital, PHL = made later in the Public Health Laboratories. This gives an idea of the cytolysis (22) characteristic of poliomyelitis specimens.

The syphilis group, which was the largest, containing 240 individual specimens, showed, in general, intermediate RNA-ase activity. One sample from a neurosyphilitic, jaundiced during fever therapy, exhibited the highest value (Table I, group D, and Table II, group IV).

The miscellaneous neurological samples occasionally showed high values. A specimen from a case of genuine epilepsy exhibited exceptionally high RNA-ase activity (46%). Symptomatic convulsions rarely gave values over 20%. Brain tumor specimens ranged from 9 to 24%. Vascular disorders ranged from 15 to 27%, with intracerebral thrombosis showing the highest values. Spinal disc lesions gave values of 15 to 21%. Polyneuritis and facial palsy (six cases) ranged from 11 to 24%, hydrocephalus samples from 20 to 25%, post-traumatic syndromes from 24 to 34%. The values for the 25 psychiatric cases were: 16 to 23% in cases of schizophrenia, and 1%, 6%, 7%, 22% respectively in single cases of anxiety neurosis, hysteria, moronity, and senile psychosis due to brain atrophy (Table I, group E, and Table II, group V).

TABLE I

DETAILED ANALYSIS OF 800 RESULTS OF RNA-ase IN THE CSF

Diagnosis	No. of specimens	Range of % of depolymerized substrate	Over-all range, %
Group A			
Normal	11	0–10	0–20
	10	11–15	
	9	16–20	
Neurologically normal	18	0–10	0–30
	26	11–15	
	21	16–20	
	3	21–25	
	2	26–30	
Group B			
Meningococcal meningitis	60	0–27	0–50
Purulent meningitis	60	2–50	
Pneumococcal meningitis	35	22–27	
Influenzal meningitis	40	21–38	
E. coli meningitis	5	20–25	
Group C			
(*a*) Nonparalytic poliomyelitis	70	15–35	15–52
Paralytic poliomyelitis	36	18–52	
Bulbar poliomyelitis	30	15–37	
Polioencephalitis	4	23–27	
(*b*) Mumps encephalitis	6	13–20	7–31
Lymphocytic meningitis	4	7–31	
Group D			
Primary syphilis	30	0–24	0–39
Secondary syphilis	20	0–12	
Early latent syphilis	35	0–24	
Late latent syphilis	15	0–20	
Neurosyphilis	80	0–39	
Tabes	30	2–21	
Paresis	20	2–28	
Taboparesis	10	10–24	
Group E			
(*a*) Convulsive disorders:			
Genuine epilepsy	16	20–46	0–46
Symptomatic convulsions	24	0–23	
(*b*) Brain tumors	25	0–22	0–27
(*c*) Psychiatric cases	25	1–23	
(*d*) Hydrocephalus	4	20–25	
(*e*) Vascular diseases	6	10–24	
(*f*) Post-traumatic syndromes	10	10–24	

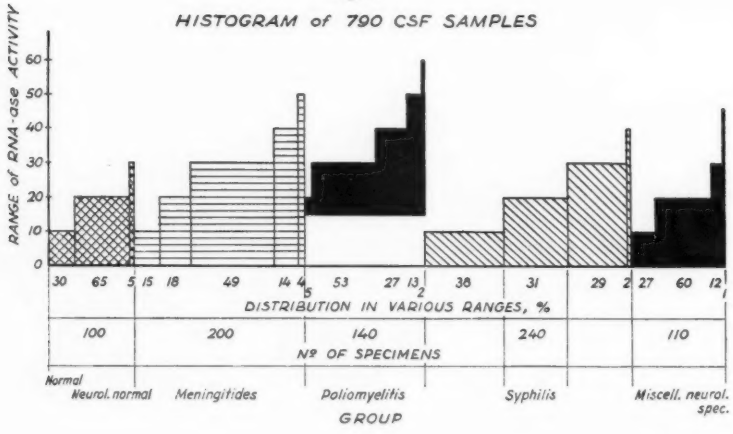
TABLE II

SELECTED CASES OF RNA-ase IN THE CSF

Name	Diagnosis	Notes	Substrate % depolymerized	
Group I—Normals, neurologically normals: 100 cases				
A.B.	Normal case	Neg. CSF findings	1.5	
M.L.	Pharyngitis	Neg. CSF findings	0	
E.S.	Gastroenteritis	CSF: proteins 62 mgm. % cells	1.2	
F.G.	Infectious mononucleosis	Neg. CSF findings	28	Exceptions
M.S.	Myositis	Neg. CSF findings	30	Exceptions
Group II—Meningitides: 200 cases				
J.M.	Meningococcal meningitis	200 cells Pá pos.	15	
G.L.	Meningococcal meningitis	1160 cells Pá pos.	27	
C.S.	Pneumococcal meningitis	600 cells Pá pos.	25	
D.D.	Pneumococcal meningitis	1800 cells Pá pos.	38	
J.E.	Influenzal meningitis	1400 cells Pá pos.	31	
R.C.	Purulent meningitis	200 cells Pá pos.	12	
D.B.	Purulent meningitis	4500 cells Pá pos.	50	
P.S.	*E. coli* meningitis	8200 cells Pá pos.	25	
Group III—Poliomyelitis and other virus diseases: 150 cases				
		*H **PHL		
N.B.	Nonparalytic poliomyelitis	39 0 cells Pá trace	29	
N.N.	Nonparalytic poliomyelitis	11 0 cells Pá ++	32	
B.L.	Nonparalytic poliomyelitis	170 70 cells Pá +	35	
M.O.	Paralytic poliomyelitis	15 0 cells Pá ++	36	
H.M.	Paralytic poliomyelitis	176 100 cells Pá ++	36	
M.K.	Paralytic poliomyelitis	247 168 cells Pá trace	52	
M.T.	Bulbar poliomyelitis	24 7 cells Pá +	37	
M.M.	Bulbar poliomyelitis	12 0 cells Pá ++	33	
E.C.	Bulbar poliomyelitis	2 0 cells Pá +	29	
J.W.	Polioencephalitis	33 10 cells Pá ++	23	
B.S.	Lycitic meningitis	231 — cells Pá trace	31	
Group IV—Neurosyphilis: 240 cases				
E.E.	Primary syphilis	Wass. R. Ser. pos., CSF neg.	6	
E.D.	Secondary syphilis	Wass. R. Ser. pos., CSF neg.	12	
J.H.	Early latent syphilis	Wass. R. Ser. pos., CSF neg.	24	
S.H.	Late latent syphilis	Wass. R. Ser. pos., CSF neg.	7	
M.S.	Neurosyphilis	Wass. R. Ser. pos., CSF neg.	39	Jaundiced
J.M.	Tabes	Wass. R. Ser. neg., CSF mod. pos.	2.4	
W.M.	Taboparesis	Wass. R. Ser. 2 pos., CSF mod. pos.	24	
H.D.	Paresis	Wass. R. Ser. neg., CSF neg.	28	
Group V—Miscellaneous neurological conditions: 110 cases				
L.J.	Idiopathic epilepsy	Neg. findings in CSF	46	
R.S.	Post-traumatic syndrome	Neg. findings in CSF	24	
A.G.	Schisophreny/catatonic	Neg. findings in CSF	23	
N.D.	Neurofibromata	Neg. findings in CSF	20	
E.M.	Chromophobe adenoma	Prot. 78 mgm. %	17	
M.H.	Intervertebr. disc. lesion	Prot. 50 mgm. %	21	
B.H.	Status p. encephalitis	58 cell prot. 27 mgm. %	46	

* *H = Cell count in hospital.*
** *PHL = Cell count in Public Health Laboratories.*

Fig. 1



A histogram is included for graphic illustration of all the results with a number of specimens, which shows range of activity and percentage distribution of the values within the groups. Only 5% of the neurologically normal specimens range from 20 to 30% substrate depolymerized, whereas 49% of the meningitis, 53% of the polio, 29% of the syphilis, and 14% of the miscellaneous neurological specimens are within this range. The distribution of specimens in the range of 30 to 40% substrate depolymerized is even more suggestive: none of the normal specimens appear in this range; 14% of the meningitis cases, 27% of the poliomyelitis, 2% of the syphilis, and 1% of the miscellaneous neurological cases. Four per cent of the meningitis, 13% of the poliomyelitis, and only 1% of the miscellaneous neurological samples fall in the group between 40 and 50% substrate depolymerized. Poliomyelitis alone shows activity in the range between 50 and 60%. The characteristic lack of low values encountered in the CSF of poliomyelitis cases is clearly shown in the histogram.

In regard to the kinetics of the enzyme system under the conditions of our assays, the following observations were made. The enzyme was assayed through a wide pH range (5.5 to 8.3) and as observed also by Kunitz and others (17, 27) showed maximum activity at about pH 7.7. It was active between 37° and 70° C., with a temperature optimum of about 60° C. No linear relationship between the time factor and enzyme concentration was found. Spontaneous hydrolysis of substrate increased with the increase of alkalinity but was never excessive, and the value of the substrate control, referred to above in the description of methods, was always considered in the calculations. Mg-ion was assayed as activator in the reaction mixtures, but the optimal molar strength was not calculated, because in preliminary assays the enzyme was found to be not very sensitive to change in the concentration of magnesium. This is in agreement with the experience of others (27). At

the optimal hydrogen-ion concentration the enzyme was quite resistant to heat inactivation, but was readily inactivated at the extreme pH ranges. Sodium fluoride in $N/1000$ end concentration inhibited RNA-ase activity, whereas arsenate had no effect in the same concentration. Repeated freezing and thawing resulted in a loss of about 10 to 15% of the RNA-ase in a series of CSF's in parallel assays. The relationship between cell count, protein concentration, and RNA-ase activity is illustrated in Table III, and is further discussed.

TABLE III

RELATIONSHIP BETWEEN RNA-ase ACTIVITY AND CELLS AND PROTEINS

Number	Group	Cells	Proteins	RNA-ase activity
100	Normal, neurol. normal	None or low	None or low	None or low
200	Meningitis	Very high	High	High
140	Poliomyelitis	Low or moderately high	Moderately high	Very high
16	Epilepsy	None or low	None or low	High or moderate
6	Cerebr. thrombosis	High	Trace	Moderate
2	Hydrocephalus	None or low	Very high	Moderate

The RNA-ase activity of a small number of specimens from each etiological group was definitely confirmed by other methods. Measurement of diffusibility through semipermeable membrane, of acid precipitation, and of changes in ultraviolet light absorption (17, 18) agreed very well with the total acid soluble phosphorus findings in the same systems.

Discussion

It is evident from our reported assays of nuclease activity in 800 cerebrospinal fluids that this enzyme is very commonly present in variable amount, especially under pathological conditions. If one considers the dilute nature of the CSF and the small volume used in the assays at a temperature and pH simulating body conditions, but far from the optimum (17), the RNA-ase activity is striking. The highest value obtained in our series was 52%. Dubos (5), using concentrated enzyme, and Kunitz (17) using crystalline RNA-ase under ideal conditions, found that about 60% of the substrate was depolymerized in a very short time.

In normal samples, little RNA-ase activity was present, ranging from 0 to 10%. The difficulty of obtaining definitely normal CSF's forced us to include 70 samples from neurologically normal individuals. The majority exhibited low values; the remainder ranged to 20%. Here we were dealing with various

pathological, mostly febrile, conditions that may enhance the enzyme activity. Two of the exceptional values were not very high, but three specimens were definitely over the empirical base line (see Results). All three were poliomyelitis suspect cases in the poliomyelitis season, but the final diagnosis of the hospital staff that these were not poliomyelitis was accepted for our grouping. One specimen, if the diagnosis of myositis is correct, presents a very interesting problem, namely high RNA-ase activity (30%) during inflammation of the muscles.

In meningitis cases the highest activity was found in CSF's with a high cell count. The relationship was definite but not absolute. The same could be said about the protein concentration and RNA-ase activity. This is true in the acute and subacute stage of the disease, when the activity shows a peak, compared with convalescence. In about 18 cases we had occasion to make determinations in the same individuals in different phases of the disease. The higher values in the early stage evidently depend on the inflammatory reactions and/or on the permeability of the barrier (12, 13). The polymorphnuclear leucocytes, according to Dubos (27), and hemolytic streptococci, according to McCarty (27), do contain RNA-ase.

If we review all the assays, it is striking that the highest activity was uniformly present in poliomyelitis specimens. In 80 out of 140 polio samples we had high values, compared with 22 out of 200 in meningitis and 3 out of 240 syphilis specimens. All the poliomyelitis samples were taken with only one lumbar puncture in the acute or subacute stage of the disease during chromatolysis of the motoneurons (9). The relationship is not only chronological; there is definite evidence of intense nucleic acid and enzyme changes during experimental chromatolysis (8). Bodian postulated the presence and activity of nucleases in neuronophagy (1) and in experimental chromatolysis, without demonstrating them (8). The increase of phosphatases (2) however does not mean the increase of nucleases.

The Nissl bodies consist of ribonucleoproteins (21), and lend themselves as substrate for this enzyme activity. Different authors (3, 8) demonstrated loss of stainability (similar to chromatolysis) of the Nissl bodies of normal cord slides, when incubated in crystalline RNA-ase solution.

Pathological postulates, based on experimental evidence, are supported by the present clinical investigation, which demonstrates quantitatively the high RNA-ase activity in poliomyelitis at the time of chromatolysis. These findings are of special significance because they suggest some direct connection between chromatolysis and RNA-ase activity. Other enzymes described in these CSF's (4, 7, 12) may take part, but the RNA-ase has a primary importance. In regard to the origin of the enzymes of the CSF, we refer to the general discussion of this topic by Kaplan and others (12, 13).

The relationship between RNA-ase activity and cell count in the poliomyelitis group is not strict. The highest value (52%) was found in a CSF with 247 cells, but another paralytic polio specimen with 1044 cells exhibited only 18% change. The same holds true for the relationship with the protein

concentration, thus emphasizing the essential difference between the meningitis and poliomyelitis groups. In a previous publication (16) we described a lack of autolysis of phosphate compounds in the CSF of poliomyelitis patients and explained this finding tentatively by specific inhibition. The high RNA-ase activity in the same specimens is rather interesting. Cytolysis (22) and nuclease activity show the same lack of strict parallelism (Table II, group III), suggesting that the RNA-ase does not necessarily originate from the disintegration of leucocytes.

The only very high value in the syphilis group (jaundiced patient during fever therapy) indicates the complexity of factors which may originate or influence this enzyme activity. Epilepsy cases show complete independence of RNA-ase activity from both cells and proteins (Table III). Increased permeability would permit the entry of enzymes from the blood and/or the nerve tissue (12, 13). The only data about nucleases in the CSF have been described in convulsive disorders and in post-traumatic syndromes (25, 26). Our results corroborate these findings.

Although the diversity and the small numbers of psychotics do not warrant any conclusions, the higher values found in brain atrophy and in catatonia are noteworthy. Our investigations of desoxyribonuclease and nucleotidase of the CSF are proceeding, and we hope to publish our results soon.

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ON THE NUTRITIONAL REQUIREMENTS OF YOUNG CHILDREN WITH PARTICULAR REFERENCE TO CALCIFICATION[1]

By Thelma Allen, Ada V. MacLeod, and E. Gordon Young

Abstract

Two nutritional surveys have been carried out on 158 children of one to six years in Halifax, including dietary records, anthropometric measurements, roentgenograms of the bones of the hand, and laboratory tests. Observations were repeated after six months. Deficiencies in calories, calcium, and vitamin D occurred most frequently on the basis of the Canadian dietary standard. Seventy-eight per cent of the children received less than 1 gm. of calcium per day. The ratio Ca : P averaged 0.77. The consumption of vitamin D varied widely and was less than 400 I.U. in 47% in the first survey and 84% in the second. The average intake was less than 200 I.U. per day. From data of repetitions it was apparent that half of the children showed adequate growth and calcification on substandard diets while 30% did not. It was evident that normal maturation of bone could take place on intakes of vitamin D of 25 to 150 I.U. and of calcium of less than 1 gm. Calcium was adequate at 0.2 to 0.7 gm. per day in some cases but not in others.

Introduction

This study was commenced in 1945 when the scientific literature contained relatively few papers on the dietary requirements of infants and children. The baby under one year of age has been very carefully studied. Extensive anthropometric measurements of growing children are available but in few instances related to diet (1, 3, 9, 28, 33, 35). Within the past decade moderately accurate methods of analysis for vitamins have been developed so that an assessment of diet in relation to growth in children is now possible.

Surveys based on questionnaires or the use of 'average' portions cannot be considered as contributing reliable *quantitative* data to our knowledge of dietary requirements for normal growth. The classical work of Holt and Fales (13) on a selected group of 100 children between one and 16 years of age has provided data in terms of energy, protein, fat and carbohydrate. These results have been extended in nutritional surveys of wider scope by Youmans, Patton, and Kern (37) in Tennessee, by Beal, Burke, and Stuart (2) in Boston, and by Scobie, Burke, and Stuart (26) in Newfoundland. The most extensive survey of children's diet has however been published by Widdowson (36) in England. In this study 916 children between 1 and 18 years of age were examined and dietary records during one week were obtained between 1935 and 1939. A résumé of the evidence contributing to the selection of levels of requirements is already available (19). Many of the values set down as recommended dietary allowances are without adequate scientific foundation, particularly for vitamins and especially for vitamin D. While 400 to 800 I. U. per child per day are recommended the factual basis for these figures is meager (14, 19, 23, 30).

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The purpose of the surveys to be described was to obtain dietary data for young children from one to six years of age and to correlate these findings with the physical condition and rate of growth by repeating the examination at intervals of approximately six months. Anthropometric and radiographic measurements and a limited number of laboratory tests were also made. From the data it was hoped to be able to deduce levels indicative of amounts of essential nutrients which would or would not permit of normal growth, ossification, and good physical condition.

Procedure

Two nutritional surveys have been made on children of preschool age, between one and six years. The first was carried out between 1945 and 1947 on 55 children from the wealthier class in Halifax, N.S. The second was conducted between 1949 and 1951 on 101 children from the poorest class in the same city with a population of approximately 100,000.

The food consumed by the children was weighed on standard dietetic scales. The investigator made three visits to the family during the course of the week. The child then came to the Dalhousie Public Health Clinic as soon as possible. Physical examinations were made by a pediatrician and records kept on standard forms. Anthropometric measurements included body weight, standing height, pelvic girth, and chest circumference by the methods of Vickers and Stuart (33). Chest circumference was measured in the sitting position. X-radiographs of the bones of the wrist were taken in the Department of Anatomy on many of the children to determine age of maturation by the method of Todd (32).

A specimen of urine was tested routinely for the presence of protein and of reducing sugar. Drops of blood were taken for red and white cell counts and for the estimation of hemoglobin by the Newcomer method.

The data on food consumption were calculated in terms of calories, protein, fat, carbohydrate, calcium, phosphorus, iron, and vitamins (A, B_1, B_2, C, D, and niacin), from standard tables (5, 8). They are expressed as the mean of the consumption for one day from the observations during one week. Differences between sexes are not statistically significant up to the age of seven in any category according to the evidence of Widdowson (36) and we have confirmed this conclusion. No differentiation has therefore been made between boys and girls in our calculations. Meredith (18) has made a careful analysis of stature in this regard.

The distribution of the children in the two surveys in terms of age and sex is shown in Table I. The mean values for the anthropometric measurements of the two groups are listed in Table II. It is apparent that the children in group II were smaller than those in group I. The dimensions in group I are almost identical with the values obtained by Vickers and Stuart (33), taken at the half-year, for children of North European stock in Boston. They are slightly lower than the values for weight and height in Cleveland, Ohio, of

TABLE I

DISTRIBUTION OF CHILDREN IN RELATION TO AGE AND SEX

Age, yr.	Survey 1		Survey 2		Total
	♂	♀	♂	♀	
1 – 2	4	10	7	7	28
2 – 3	7	6	6	5	24
3 – 4	8	5	10	14	37
4 – 5	2	5	9	7	23
5 – 6	5	3	9	10	27
6 – 7	—	—	11	8	19
Totals	26	29	52	51	158

TABLE II

ANTHROPOMETRIC MEASUREMENTS

Age, yr.	Weight, kgm.			Height, cm.			Chest circumference, cm.			Pelvic girth, cm.		
	1	2	*S**	1	2	*S**	1	2	*S**	1	2	*S**
1 – 2	11.5	10.6	*11.5*	79.5	78.3	*81.9*	48.4	48.1	*49.4*	15.4	12.0	*13.6*
2 – 3	14.5	12.5	*13.7*	91.5	85.5	*91.1*	50.7	49.5	*51.7*	15.0	13.5	*15.1*
3 – 4	15.2	14.4	*15.7*	95.5	93.8	*98.5*	51.6	51.1	*53.1*	16.0	14.7	*16.3*
4 – 5	17.3	17.0	*17.6*	104.0	101.9	*105.2*	52.0	53.4	*54.2*	17.0	15.5	*17.1*
5 – 6	19.9	19.5	*19.7*	112.5	108.7	*112.0*	55.4	53.4	*55.6*	18.4	15.0	*18.1*
6 – 7	—	20.8	*21.7*	—	116.3	*117.8*	—	49.1	*57.1*	—	15.9	*19.1*

1 – *First survey.*
2 – *Second survey.*
* *Vickers and Stuart (33).*

Simmons (28) and rather irregular as compared with the values given by Widdowson (36) for English children. There are no comparable figures for Canadian children of this age.

Hematological values are presented in Table III. The average for the hemoglobin levels of 55 children on initial examination was 12.4 gm. per 100 ml. but if subsequent repetitions are included this becomes 12.7 gm. from 92 analyses. The range was from 10.6 to 14.1. A comparable series of observations was made on 319 children, one to six years of age, in various

parts of Canada by Pett and Ogilvie (24). The average was 12.5 and the approximate range 10 to 15. Figures obtained by Widdowson (36) in England on a similar group of 25 children averaged 12.5. There is thus remarkably close agreement. The red cell counts are slightly higher than those usually quoted in textbooks on pediatrics at 4.5 to 4.8 $\times$ 10^6. The white cell counts in Table III are slightly lower than those currently quoted at 8 to 9 $\times$ 10^3 (4).

TABLE III

HEMATOLOGICAL VALUES—SURVEY I

Age, yr.	Hemoglobin, gm./100 ml.	R.B.C., cells $\times$ 10^{-6}/mm.^{3}	W.B.C., cells $\times$ 10^{-3}/mm.^{3}
1 – 2	12.5	5.0	7.5
2 – 3	12.1	5.0	7.2
3 – 4	12.4	5.2	6.7
4 – 5	12.4	5.0	7.0
5 – 6	12.9	5.4	6.0
Av.	12.4	5.1	7.0

The consumption of food in terms of essential nutrients for both surveys is presented as averages per child per day in Table IV, together with requirements as calculated from the current Canadian dietary standard (6). It is apparent that the children of survey I were consuming more food per child than those in survey II. Their greater weight and height are shown in Table II. As compared with the standards, deficiencies in calories, calcium, and vitamin D are evident in most categories according to age. The intakes of other essential nutrients are close to, or far in excess of the standards. If compared with the recommended dietary allowances of the U.S.A., which bracket children of one to three years and four to six years, it is frequently apparent that the consumption of the children in the lower year is less than that recommended, about equal in the intermediate year and higher in the upper year. Intakes lower than recommended are indicated in calcium and occasionally in calories, phosphorus, iron, thiamine, ascorbic acid, niacin, and vitamin D. This applies particularly to the children in survey II. The intakes of children in survey I were deficient only in calcium and occasionally in calories and niacin.

The variation in the levels of intake is frequently very wide both for a particular group and a single child. In order to present this fact in the most useful form, the variation, inclusive of 80% of the children in each group, selected towards establishing normal distribution, is shown in Table V for calories, calcium, phosphorus, and vitamin D. It is evident that the widest variation occurs in the consumption of vitamin D and the second in calcium.

TABLE IV

AVERAGE CONSUMPTION OF ESSENTIAL NUTRIENTS PER CHILD PER DAY CANADIAN STANDARD BASED ON AVERAGE WEIGHTS FOR AGE CATEGORIES

Age, yr.	No. of survey and children	Cal.	Protein, gm.	Fat, gm.	Carbo., gm.	Ca, gm.	P, gm.	Fe, mgm.	Vitamins					
									A, I.U.	B_1, mgm.	B_2, mgm.	C, mgm.	D, I.U.	Niacin, mgm.
I – 2	I – 14	992	38	36	121	0.90	0.92	12.4	13,100	0.77	1.57	51	1,525	4.6
	II – 14	735	30	30	90	0.47	0.61	5.6	5,580	0.45	0.80	34	1,310	3.5
	S	*1150*	*36*	*32*		*1.0*	*1.0*	*6*	*700*	*0.35*	*0.58*	*30*	*400*	*3.5*
2 – 3	I – 13	1,251	42	46	166	0.62	0.81	9.0	10,750	0.79	1.48	58	1,190	6.1
	II – 11	952	36	41	108	0.64	0.72	4.5	5,126	0.54	1.17	40	365	4.7
	S	*1250*	*37*	*36*		*1.0*	*1.0*	*6*	*1000*	*0.40*	*0.65*	*30*	*400*	*4.0*
3 – 4	I – 13	1,387	49	51	190	0.83	1.06	10.5	7,840	0.81	1.46	63	352	7.5
	II – 24	1,177	38	47	143	0.53	0.83	6.3	4,187	0.52	0.95	31	164	5.3
	S	*1380*	*38*	*42*		*1.0*	*1.0*	*6*	*1100*	*0.45*	*0.75*	*30*	*400*	*4.5*
4 – 5	I – 7	1,291	48	56	164	0.84	1.06	8.8	4,030	0.81	1.39	89	586	6.8
	II – 16	1,211	42	48	151	0.54	0.85	6.2	4,544	0.57	0.93	42	165	6.1
	S	*1500*	*40*	*44*		*1.0*	*1.0*	*6*	*1300*	*0.50*	*0.80*	*30*	*400*	*5.0*
5 – 6	I – 8	1,518	52	55	210	0.83	1.14	9.7	12,590	0.93	1.55	82	1,006	6.9
	II – 19	1,527	50	58	194	0.66	1.00	9.8	5,368	0.65	1.18	37	285	7.3
	S	*1650*	*42*	*47*		*1.0*	*1.0*	*6*	*1400*	*0.52*	*0.85*	*30*	*400*	*5.2*
6 – 7	II – 19	1,586	53	64	195	0.82	1.10	8.1	3,885	0.75	1.44	61	334	7.2
	S	*1800*	*44*	*50*		*1.0*	*1.0*	*6*	*1500*	*0.55*	*0.90*	*30*	*400*	*5.5*

TABLE V

VARIATION INCLUSIVE OF 80% OF CHILDREN

Nutrient	Survey	Age. yr.					
		1 – 2	2 – 3	3 – 4	4 – 5	5 – 6	6 – 7
Calories	I	750 – 1164	828 – 1457	1023 – 1525	1139 – 1469	1076 – 1668	—
	II	617 – 1112	765 – 1159	531 – 1503	958 – 1425	953 – 1988	1217 – 1974
Calcium (gm.)	I	0.55 – 1.30	0.35 – 0.90	0.53 – 1.13	0.57 – 0.90	0.65 – 1.06	—
	II	0.24 – 0.83	0.23 – 0.89	0.23 – 0.99	0.21 – 0.80	0.25 – 1.05	0.56 – 1.16
Phosphorus (gm.)	I	0.64 – 1.22	0.50 – 1.22	0.68 – 1.48	0.77 – 1.30	0.84 – 1.29	—
	II	0.48 – 1.05	0.47 – 0.84	0.28 – 1.28	0.50 – 1.00	0.52 – 1.33	0.83 – 1.31
Vitamin D (I.U.)	I	85 – 2734	36 – 1332	26 – 821	21 – 1516	25 – 1665	—
	II	27 – 119	13 – 343	15 – 153	32 – 184	20 – 425	32 – 1468

Calories

The results are expressed graphically in Fig. 1 in which it may be seen that the levels of energy of the food consumed by children in survey I corresponded closely to the standards and were definitely above those of survey II by 200 Cal. or more, in the lower categories. At one to two years there is an apparent departure from the linear relationship of age to calories. This coincides with the normal curve of growth. The results of Virtue and McHenry (34) on about 400 children in Toronto, Ont., are plotted as they also represent a level of intake lower than the standards. The results of Beal, Burke, and Stuart (2) in Boston and those of Scobie, Burke, and Stuart (26) for Newfoundland are higher than ours at all ages.

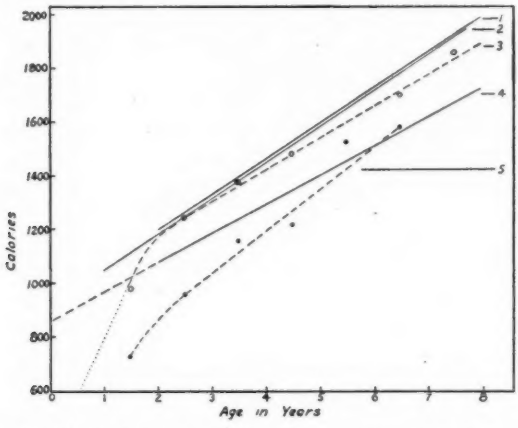

FIG. 1. Calories in relation to age: (1) U.S.A. recommended dietary allowances. (2) Canadian dietary standard, (3) our results in survey 1, (4) results of Virtue and McHenry (24), (5) our results in survey 2.

Protein

The consumption of protein in survey I was slightly higher than in survey II but both are in astonishingly close agreement with previous results in England (36) and in Newfoundland (26). They tend to be a few grams higher than the Canadian standards. A departure from a linear relationship to age is notable at one to two years. Protein accounted for between 13 and 16% of the total calories.

Fat

Fat consistently provided between 34 and 36% of the total calories and was consumed in amounts somewhat above the standards, based on 25% of the total calories.

Calcium and Phosphorus

As compared to the present standard of one gram the intake of calcium in both surveys was definitely low. This was particularly true for the children

in the second group at all ages. The intake of phosphorus was also lower than the standard at most ages in the second group. These facts are most clearly shown in the variations listed in Table V. The ratio Ca : P varied between 0.73 and 1.0 in the first group and between 0.64 and 0.89 in the second group, with an over-all average of 0.77. The percentage of the total number receiving less calcium than the standard was 78. This fact is of considerable importance in the consideration of the degree of maturation from the roentgenograms discussed below. In all comparable groups studied by Widdowson (36) and by Scobie *et al.* (26) the average intakes were less than one gram of calcium.

Iron

The consumption of iron may be considered as adequate in relation to the standard with few exceptions and in no individual was it less than 5 mgm. in survey I. It was notably high in children of one to three years because of the use of special foods rich in iron. In survey II 50% of the children were ingesting less than 6 mgm. and it frequently fell to 4 mgm. or less. Unfortunately we have no values for levels of hemoglobin in this group.

Vitamin A

There is a notably high consumption of this vitamin regardless of the factor adopted for the equivalence of β-carotene and vitamin A. In the children of survey I this was due to the frequent use of fish liver oils. The range of individual intake was very wide. Our figures are higher than those of the English and Newfoundland surveys.

Vitamin B_1

Based on a standard allowance of 0.3 mgm. per 1000 Cal., no deficiency in either group was apparent in the averages although about 25% of the children in survey II were substandard in their intakes.

Vitamin B_2

On the basis of a standard allowance of 0.5 mgm. per 1000 Cal., the same adequacy of intake as for B_1 is evident. Nevertheless about 20% of the children in survey II were below the standard.

Vitamin C

The agreement of the averages in survey II with the standard is close in several categories and a substandard consumption occurred in 36% of the children of this group.

Vitamin D

The sharpest contrast between our two surveys is to be found in this vitamin. This is attributable to the general use of fish liver oils in group I and the lack of them in group II except for infants of one to two years. The individual variation was very wide as can be seen in Table V. Children receiving this

supplement often ingested over 1000 I. U. per day while children without it frequently ingested less than 100 I. U. In survey I 47% were receiving less than 400 I. U. and in survey II it was 84%. On a distribution curve the great mass of children were below an intake of 200 I. U. This fact, especially when considered in relation to growth and calcification, is probably the most important finding of the survey. It agrees with the results of Widdowson (36) for English children, although our averages in group II are somewhat higher than hers. Few surveys have thus far included this vitamin and these facts may be of help in establishing a proper physiological level as a standard. It is significant that no case of active rickets was observed during physical examination.

Niacin

The average consumption was adequate based on a standard of 3.0 mgm. per 1000 Cal. Of the children in survey II, 36% were below the standard.

Nutrient Intake per Kgm. of Body Weight

Consumption of the essential nutrients in both surveys has been calculated per kilogram of body weight, except for vitamins B_1, B_2, and niacin which have been expressed per 1000 Cal., as listed in Table VI. The results for the children in group I are still somewhat higher than those of group II especially for calcium, iron, vitamins A, B_1, B_2, C, and D. It is evident that the high nutritional plane of the first year of life is carried over into the second. No particular drift of any nutrient is evident with increasing age, although this has already been established for calories and protein by Holt and Fales (13). Our results with protein suggest that 3.0 gm. per kgm. is adequate at one to three years and 2.5 gm. per kgm. at four to six years, in exact agreement with the current Canadian standard (6).

The intake of fat at approximately 3.1 gm. at all ages examined is close to the value of 3.3 to 3.6 gm. recorded by Holt and Fales (13).

The consumption of calcium was 46 $\pm$ 8 mgm. and of phosphorus 61 $\pm$ 10 mgm. per kgm. The former value is to be compared with the level of 50 mgm. proposed as desirable by Sherman and Hawley (27), of 45 to 58 mgm. found by Daniels *et al.* (7), and of 32 to 44 mgm. determined by Outhouse *et al.* (21).

Iron at 0.55 $\pm$ 15 mgm. is close to the figure of 0.50 mgm. found by MacKay (16) and Stearns and Stinger (31). Other values in the literature range from 0.32 to 0.60 mgm. (14).

The consumption of vitamin A was high in all subgroups and well above recommended allowances.

Relative to the Canadian standard for B_1, B_2, and niacin at 0.3, 0.5, and 3.0 mgm. per 1000 Cal. respectively, the average intakes of the Halifax groups were more than adequate.

Vitamins C and D have not been correlated with either body weight or the energy value of the diet. For C there appears to be a certain uniformity to

TABLE VI

INTAKE OF ESSENTIAL NUTRIENTS PER KGM. OF BODY WEIGHT OR PER 1000 CAL.*

Age, yr.	Survey	Cal.	Protein, gm.	Fat, gm.	Ca, mgm.	P, mgm.	Fe, mgm.	Vitamins					
								A, I.U.	B_1*, mgm.	B_2*, mgm.	C, mgm.	D, I U.	Niacin*, mgm.
1 - 2	I	86	3.3	3.2	74	81	1.04	1135	0.78	1.59	4.4	133	4.6
	II	71	2.8	3.0	46	58	0.53	548	0.62	1.09	3.3	134	4.8
2 - 3	I	85	2.9	3.2	45	55	0.62	747	0.63	1.18	4.0	82	4.9
	II	78	3.0	3.4	52	59	0.37	422	0.56	1.21	3.2	30	4.9
3 - 4	I	91	3.3	3.3	54	74	0.69	497	0.58	1.05	4.7	21	5.4
	II	82	3.1	3.3	37	58	0.43	290	0.45	0.83	2.2	6	3.4
4 - 5	I	75	2.8	3.1	50	64	0.51	531	0.63	1.08	5.1	30	5.3
	II	71	2.5	2.9	32	51	0.36	295	0.48	0.77	2.5	10	5.0
5 - 6	I	76	2.6	2.7	40	56	0.51	618	0.61	1.02	3.9	49	4.5
	II	87	2.8	3.3	38	57	0.56	305	0.42	0.77	2.2	16	4.8
6 - 7	II	76	2.6	3.1	40	53	0.39	174	0.47	0.91	3.0	16	4.6
Av.			2.9	3.1	46	61	0.55	506	0.57	1.05	3.5	48	4.7

weight at 3.5 ± 1.0 mgm. The variation in D on this basis is very wide. Assuming a figure of 350 I. U. for the infant (14), this would be distributed as follows on a basis of per kgm.

100 I. U./kgm. at birth		
34	kgm.	1 year
23	kgm.	3 years
18	kgm.	5 years

Our results show definite inadequacy in some subgroups on this basis and a more detailed analysis is given below. Glasser *et al.* (11) found that 100 I. U. were sufficient for normal growth and that 200 I. U. were as efficacious as 400 I. U. in promoting retention of calcium in premature infants. On the basis 200 I. U. the distribution per kilogram of body weight would be

17 I. U. for 1 – 2 yr.
13 I. U. for 3 – 4 yr.
10 I. U. for 5 – 6 yr.

Radiological Examinations

Roentgenograms of the bones of the wrist of some of the children in the first survey and most of the children in the second survey were taken by the method of Todd (32). One person, with considerable experience in this field, made the assessment of degree of maturation against the standards of Greulich and Pyle (12) without knowledge of the chronological age. On first examination of the 56 children, 68% were below the standard densities by 2 to 35 months and 30% were above by 1 to 49 months. The average number of months for the subnormal group was − 9.5 and for the supernormal group + 9 months. The latter figure is affected by one particularly high value of + 49. Deleting this value, the average becomes + 6.

The extent of what may be termed normal discrepancy between chronological and assessed age is undetermined at present. For middle and later childhood Todd (32) was inclined to regard a discrepancy of more than six months as indicative of retarded or accelerated maturation. Accepting this figure tentatively, we find 29% of our group retarded and 14% accelerated. On second examination nine children of 32 examined changed their relative rating by ± 3 months or more; six lost an average of five months and three gained an average of five months. This group will receive closer analysis in relation to diet below.

Repetitions

For the reason that the children in survey I were better fed than in survey II and few radiological examinations were made on them, analysis of the repetitions has been confined to the second survey.

There were 39 repetitions of the standard procedures of the survey done on the group of 63 children (i.e. 62%), after the lapse of approximately six months.

This group provides a better criterion of the adequacy of the diets in comparison with the standards. It furnishes data on which to check the standards themselves in relation to growth and development. The record—physical, dietetic, and radiographic—of each child must be considered individually. Physical development and state of health have been assessed from the records.

In only about one fifth of the children was the usual standard of 1 gm. of calcium attained, whereas about 60% reached this level for phosphorus, and about 70% the standard intake for iron. About one third ingested 400 I. U. or more of vitamin D during one or other period of observation. In the group of 39, about 15 showed a delayed state of maturation beyond the six months allowed for normal variation as indicated above (i.e. about 40%).

Certain important conclusions may be drawn from the details of individual cases as analyzed. Of the 39 cases, 3 should be deleted for various reasons. Of the remaining 36, only 6 conformed to normal standards of diet and growth. Some 17 showed adequate calcification and growth on substandard diets. Some 11 showed inadequate calcification or growth on substandard diets. One showed markedly retarded calcification on an apparently adequate diet. One showed a mixed picture of retarded development on a substandard diet but normal calcification over the period of observation.

It is evident that normal maturation of bone may proceed on intakes of vitamin D at 25 to 150 I. U., and of calcium and phosphorus at less than one gram. Calcium may be adequate at 0.2 to 0.7 gm. in some cases but it is apparently not adequate in others.

Discussion

Several possible sources of error enter into the validity of the data presented and these have been discussed by McHenry *et al.* (15). The period of one week (21 consecutive meals) for dietary records has been generally adopted by workers in the field of nutrition. Longer periods would be more desirable but present several added difficulties. Repetitions with the Halifax children have helped to check the accuracy of these observations, but the inherent potential errors of this type of observation remain a factor of 'indeterminacy' in nutrition. It is of importance to mention that seasonal variation, especially in the lower income groups, has been found to be remarkably small in the Canadian urban population (10).

McHenry *et al.* (15) found this variability was greatest for protein, iron, thiamine, and ascorbic acid from observations on a small, selected group of adults. The correspondence between the actual amounts of the nutrients ingested and those calculated from the tables is another important aspect of all dietary observations. Young and McHenry (38) have checked this correspondence for ascorbic acid as the nutrient most susceptible to error because of destruction during preparation and serving. The particular table of values used made a considerable difference but in the great majority of meals so assayed the actual ascorbic acid ingested was *less* than that calculated. Agreement with the food tables was good in the case of calories, fair for protein,

and poor for fat (22). Deviation in the case of calcium indicated that the actual content of the food ingested was from 86 to 94% of the calculated values (29). Our results may therefore be as much as 15% too high. For iron the actual intakes were probably fairly close to the calculated values (39). The latter tend to be greater than the actual values by about 25%.

The reciprocal relationships between calcium, phosphorus, vitamin D, and sunlight have long been recognized. The individual efficiency in the absorption and retention of calcium differs markedly. Retentions of 15 to 25% have been shown to occur on an intake of 0.9 gm. when the vitamin D supply was adequate. Retention was 40 to 90% on one of 0.2 gm. when all but a very small amount of vitamin D was obtained through the direct action of the sun's rays on the skin (7, 17, 20, 21). Normal calcification and growth of Halifax children on substandard intakes of calcium cannot be accounted for by the ingestion of standard amounts of vitamin D because this occurred in only two of 17 children in this category. The time of exposure of the children to sunlight was not known but was probably not great because of the character of their living conditions. It is more probably explained by adaptation to low intake with greater efficiency of absorption and retention of calcium phosphate. The Ca : P ration was never greater than one and never so much less than one as to be considered a factor which might affect the calcium balance.

It is recognized that the assessment of degree of maturation of the wrist bones by roentgenograms still requires further investigation and trial. Nevertheless it furnishes a useful tool, supplementary to anthropometric measurements, with which to judge the normality of growth and, indirectly, the adequacy of the diet.

The work described has been carried out as a contribution to the establishment of the range of essential nutrients which do or do not lead to 'normal' growth and development. If figures can be found which lead to standards which can be applied to the feeding of the nation's children, improvement in the national health and welfare will become possible on a more scientific basis.

Summary and Conclusions

Nutritional surveys have been carried out on two groups of children, aged one to six years, in Halifax, N.S., during 1945-47 and 1949-51. Food consumption was determined by individual weighings. Roentgenograms were taken of the bones of the hand and assessed as to age of maturation. Observations were repeated after six months, as an assessment of dietary adequacy.

The most important observations and deductions are as follows:

1. The level of hemoglobin in the blood of children in the first survey averaged 12.7 gm. per 100 ml. and the range was 10.6 to 14.1.
2. Deficiencies in calories, calcium, and vitamin D occurred most commonly in comparison with current Canadian standards.
3. Protein accounted for between 13 to 16% of the total calories and fat between 34 and 36%.

4. The ratio of calcium to phosphorus averaged 0.77 and varied between 0.64 and 1.0. Seventy-eight per cent of the children received less calcium than the standard.
5. Variation in the consumption of vitamin D was very wide depending on whether a supplementary feeding of fish liver oil was taken. In the first survey 47% received less than 400 I. U. and in the second survey it was 84%. The average intake was less than 200 I. U. No case of active rickets was noted in physical examination.
6. Essential nutrients were calculated per kilogram of body weight or per 1000 Cal. Our results confirm the standards at 3.0 gm. of protein per kgm. at one to three years and 2.5 gm. at four to six years. Fat was ingested at 3.1 gm. per kgm. at all ages. Other figures per kgm. were 46 ± 8 mgm. for calcium, 61 ± 10 mgm. for phosphorus, 0.55 ± 15 mgm. for iron.

From the repetitions it was apparent that about half of the children showed adequate growth and calcification on substandard diets while 30% of them did not. It was evident that normal maturation of bone *may* proceed on intakes of vitamin D of 25 to 150 I. U. and of calcium and phosphorus of less than 1 gm. Calcium *may* be adequate at 0.2 to 0.7 gm. in some cases but not in others.

Acknowledgments

The authors wish to record their indebtedness and gratitude to Doctors N. B. Coward, Adelaide Fleming, and H. B. Ross for the physical examinations of the children, to Professor and Mrs. D. Mainland, Doctor I. M. Murray, and Miss Blandford for radiological examinations, and to the nursing staff of the Dalhousie Public Health Clinic for courtesies extended during the course of this study.

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THE ISOLATION OF BACTERIOPHAGES FOR MYCOBACTERIA WITH REFERENCE TO PHAGE TYPING OF THE GENUS[1]

By Stephen I. Hnatko[2]

Abstract

Two hundred and eight acid-fast microorganisms were used in this investigation. Methods of isolating bacteriophages from soil, positive sputa, stool specimens, and from lysogenic strains are described. Eleven phages isolated from soil, their adaptation to other strains, and their characteristics are presented. Classification of these phages by plaque type and size, range of activity, cross-resistance tests, serological grouping, and morphology (some) are dealt with in detail. The phage preparations showed activity for 22 (44%) of 50 saprophytic strains tested. None were active against 120 human, 14 bovine, and eight avian tubercle bacilli. Though phage adsorption occurred onto some pathogenic strains it was not followed by phage multiplication and liberation. A tentative phage typing scheme is presented and discussed.

Introduction

Bacteriophages active on acid-fast microorganisms have been comparatively little studied. The first definite evidence of the existence of a bacteriophage for the acid-fast strains was that of Gardner and Weiser (9). Prior to that Steenken (16, 17) reported "spontaneous lysis" in a culture of tubercle bacilli (H37rv) associated with acidification of the medium. The filtrable lytic agent which was obtained from the "spontaneously lysed" cultures of H37rv was active on living microorganisms between a pH range of 4.2 to 5.8. Between pH 2.2 to 4.0 both heat-killed and living microorganisms showed no difference in morphology when exposed to the lytic agent. The observations were suggested to be of the same nature as those observed by d'Herelle and Bordet on other bacteria. However, though the lytic agent was determined to be filtrable it was not referred to as a bacteriophage.

Bacteriophages have now been isolated which are active against *Mycobacterium smegmatis* (9, 13, 20), *M. phlei* (12, 13), *M. paratuberculosis* (10), *M. pellegrino*, *M. fribergensis*, and *M. rabinowitschi* (13). The value of these phages as a means of classifying the mycobacteria has not been convincingly established. Penso and Ortali (13) employed five 'acid-fast' phages in their critical concentration and found only 38 of 89 saprophytic strains to be acted upon by the phages. In no instance, however, did the phages employed show activity for any of the pathogenic strains tested.

This paper describes certain 'acid-fast' bacteriophages, their isolation, adaptation, maintenance, and application to mycobacterial classification.

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Materials and Methods

Media

The basis of media for saprophytic acid-fast strains was 2% glycerol nutrient broth (12) and for the pathogenic strains was Dubos's medium (5) modified by the omission of Tween 80 and the substitution of normal horse serum and dextrose in respective final concentrations of 5 and 2% for bovine plasma fraction V. All media were solidified with agar (Bacto) prior to autoclaving.

Cultures

Two hundred and eight acid-fast cultures were used in this investigation. The age of the cultures ranged approximately from two months to two years from the date of isolation. Pathogenic strains comprised eight avian, 14 bovine, and 140 human tubercle bacilli. The human tubercle bacilli cultures ranged in age from two months to one year from the date of isolation.

Isolation of Phage from Soil

The enrichment technique followed in the recovery of phages from soil was essentially the same as described by Hnatko (12). The following acid-fast microorganisms were used to enrich 50 gm. samples of hot-bed soil semiweekly over a period of three to six: *M. smegmatis*, S_1, S_2, *M. phlei*, Ph_2, unclassified acid-fast strains 1058, 9980, 13243, and 8016, *M. tuberculosis* var. *bovis*, B_1, *M. tuberculosis* var. *hominis*, H37rv, and *M. tuberculosis* var. *avium*, A_2, A_3. The inoculum for the saprophytic strains consisted of 7 ml. of five-day broth cultures which had been centrifuged, washed, and resuspended in saline. The inoculum for the pathogenic strains consisted of 7 ml. of a 10 day culture grown in modified Dubos's medium. Two samples of soil inoculated with H37rv were incubated at 37° C. over a period of six months without revealing any phage. Eleven phages active for saprophytic acid-fast strains were isolated from soil following the above technique. Their description will appear later.

Isolation of Phages from a Lysogenic Culture

Four methods were used for detecting a lysogenic culture, that is, with the property of carrying a bacteriophage. (*a*) Actively growing broth cultures were spotted one on another on both modified Dubos's and nutrient agar plates after a method of Fisk and Wilson (8, 21); (*b*) broth culture supernatants after centrifugation were tested against surface cultures of different strains in a similar manner; (*c*) groups comprising about 15 strains were grown together for 48 hr., centrifuged, and 2 ml. of supernatant was added to a fresh culture of each of the strains within the group. Each strain was then passaged through a maximum of 10 successive cultures. After each transfer the supernatants were tested for a lytic agent by method (*b*), and (*d*) broth culture supernatants after centrifugation were spotted on heterologous cultures. Subsequently the supernatants were reinoculated with the homologous culture and the procedure was repeated till no more growth resulted. Testing for phage activity was carried out after each passage.

Of 208 acid-fast strains examined for a lysogenic culture by the four different methods, only one phage, 10142/607, was isolated successfully by method (*d*). Though plaque formation was observed in many instances no phage was isolated. Attempts to increase the titer of these lytic agents resulted in their loss.

Isolation of Phage from Positive Sputa and Stool Specimens

Forty-two positive sputa were investigated for the presence of phage. Pooled bacteria-free filtrate was obtained by adding distilled water to each specimen, shaking with glass beads to break up the lumps, and finally passing through a Buchner funnel followed by passage through a Seitz-EK filter. The filtrate was plated with 45 saprophytic and 50 pathogenic acid-fast strains and passaged daily with 15 saprophytic and weekly with 10 tubercle bacilli cultures from 6-15 passages. There was no evidence of phage activity.

Bacteria-free filtrates from 17 different stool specimens from tuberculous patients were obtained by passing a distilled water suspension through a Seitz-EK filter. Each filtrate was passaged with its homologous culture six times. The cultures used were isolated from sputa of the same patients prior to any known form of drug treatment. No evidence of phage activity was noticed.

Notation for Phage Suspensions

In this study the phages isolated from soil inoculated with *M. smegmatis* strains were designated by MS_1, MS_2, while those isolated from soil inoculated with *M. phlei* were labelled MT_1, MT_2. Phages isolated for unclassified acid-fast strains were designated by the letter M followed by the number of the culture (Table I). The method describes satisfactorily phages within the genus and species.

Any phage isolated from a lysogenic culture follows the accepted nomenclature in which the number of phage carrying the culture is given, followed by the number of the propagating strain. For example, "10142/607" means that the culture 10142 produced a phage active against 607. When one phage was derived from another as described under adaptation, the cipher describing the original preparation is followed by a stroke (/) followed by the designation of the new strain,—thus: MT_2L/Ph_2 means that phage preparation MT_2L was adapted to a new strain Ph_2 for which it originally was inactive. The new phage suspension was subsequently propagated with the new culture.

Growth, Storage and Determination of Phage Concentration

The propagation of phages with their susceptible host was carried out in nutrient broth (75 ml.) in 250-ml. Erlenmeyer flasks. Flasks were inoculated with 1 ml. of a 48 hr. culture (approx. 1.5×10^8 cells per ml.) and with appropriate phage from a stock nutrient broth suspension. The ratio was about three virus particles per cell. After 48-72 hr. incubation at 37° C. the phage–host mixture was passed through a Seitz-EK filter. The number of infective particles in a suspension was determined by the pour plate method (12) and expressed as phage per ml. The suspensions were tested for activity

and potency and stored in sealed ampules and test tubes at refrigeration temperature (6° C.). The preparations under these conditions showed a drop in titer from 10 to 35% in one year.

Results

Eleven phages were recovered from soil following the enrichment technique. Three of these have been reported previously (12). Two samples of soil were found to yield two different types of phage as is illustrated by phages MS_1 and MS_2. The inocula for these phages was *M. smegmatis*, S_1. Three phages active for saprophytic strains were isolated from soil inoculated with pathogenic strains, *M. tuberculosis* var. *bovis*, B_1, and *M. tuberculosis* var. *hominis*, H37rv. For this result no definite explanation can be given. Soils inoculated with *M. tuberculosis* var. *avium*, A_2 A_3, yielded no phage. Controls which consisted of uninoculated soils yielded no phage. Only one phage, MT_1, was specific in that it lysed only *M. phlei* strains. This phage was active on the same strains as MT_4. Both produced similar plaque type and when propagated on the same strain, Ph_2, MT_1 produced a higher titer lysate. They are probably alike.

The source of these phages, their method of isolation, and their designation is shown in Table I which should be consulted in conjunction with the previous paragraph on nomenclature.

TABLE I

Phage type	Designation	Source	Method used (enrichment)	No. of strains susceptible to phage
M. phlei	MT_1	Soil	Ph_2	5
M. phlei	MT_2L	"	Ph_1	2
M. phlei	$MT_{2a}L$	"	$Ph_1 + S_1$	2
M. phlei	MT_{2a}	"	Ph_1	2
M. phlei	$MT_{2a\,s}$	"	$Ph_1 + S_1$	2
M. phlei	MT_3	"	Ph_1	2
M. phlei	MT_4*	"	Recovered from soil filtrate	5
M. smegmatis	MS_1	"	S_1	10
M. smegmatis (?)	MS_2	"	S_1	15
M. smegmatis	MS_3	"	S_2	8
M. smegmatis	MS_4	"	H37rv	11
M. smegmatis	MS_5	"	B_1	11
M. smegmatis	MS_6	"	B_1	9
10142/607	MS_7	Strain 10142 (?)	Lysogenic culture	11
M_2/Ph_1	MS_8*	M_2** from soil	Adaptation	7
M. smegmatis	MS_9*	Soil	Soil filtrate	8
M. lacticola	ML_1*	"	"	11
M. pellegrino	MP_1*	"	"	1
M. rabinowitschi	MR_1	"	"	9
M. smegmatis	M8016	"	8016	4

* *Bacteriophages received through the courtesy of Dr. G. Penso, Istituto Superiore di Sanità, Rome, Italy.*

** *M. smegmatis phage isolated by Whittaker (20).*

Attempts were made to increase or modify the range of activity of these phages by the technique of adaptation. The success of this method depends on the chance contact of a mutant phage with a strain capable of supporting its growth and an opportunity for this phage to reproduce. Four methods were used: (*a*) cultures were 'grown' in the presence of a high concentration of phage for many passages, (*b*) plates were prepared with a high concentration of phage and varied concentrations of bacteria, (*c*) agar plates were flooded with a mixture of culture and phage. The phage dilution varied from 10^0 to 10^{-5}, and (*d*) bacteriophages which lysed more than ten strains were passaged in succession with each strain in an attempt to alter their 'surface configuration.' Any 'modified' phage as determined by the increase in range of activity was then carried through the above methods in an attempt to induce an adaptation.

By methods of adaptation three altered phages were obtained of some 34 attempts. These were M_2/Ph_1, MT_2L/Ph_1, and MT_3/S_1. They were obtained by methods (*a*), (*b*), and (*c*) respectively. Phage $MS_4/607$ was modified by methods (*d*) and (*a*). The mutants thus obtained, with the exception of $MS_4/607$, did not lose their activity for parent strains, but exhibited an augmented host range. For example, from MT_3, which lysed two strains out of 50, was obtained MT_3/S_1, which lysed six. It is of interest to note that the phages lost their original plaque type. For instance, MS_4 which produces a small haloless plaque mutated to $MS_4/607$ which produces a large halo plaque characteristic of MT phages (see Table II). Similarly MT_3/S_1 produces plaques representative of MS phages.

Attempts with a large number of phages to induce adaptation to any of 37 pathogenic strains by the above techniques resulted in indifferent success. Phages MT_1 and MT_3 produced plaques on three human tubercle bacilli strains 2288, 5592, and 5131 but these plaques could not be propagated beyond the second passage. A study of the rate of absorption of MT_3 phage to five-day cultures of human tubercle bacilli showed that the phage became adsorbed to the cells but was followed with no multiplication.

FIG. 1. Plaque formation of mutant *M. phlei* phage, MT_{2_3}, on ATCC 607; 24 hr. incubation (slightly enlarged).

FIG. 2. Plaque formation of mutant *M. phlei* phage, MT_{2a_3}, on same strain (slightly enlarged).

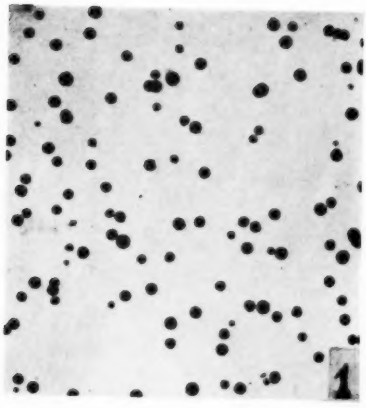
FIG. 3. Plaques of *M. phlei*, MT_2L phage, illustrating variation in plaque type; 24 hr. incubation followed by alternate refrigeration (6° C.) and incubation (24° C.). (Photograph is slightly enlarged.)

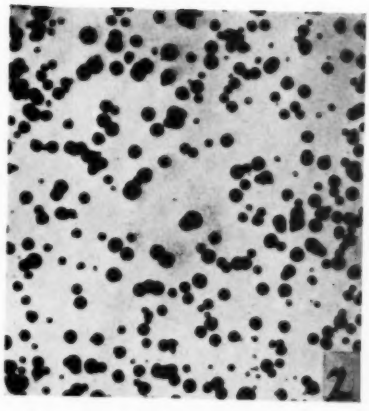
FIG. 4. Variation in plaque types of *M. phlei* phage, $MT_{2a}L$, under similar conditions as in Fig. 3. The variation between plaques is more striking here. (The above four photographs were taken by Tats Yamamoto on contrast process film.)

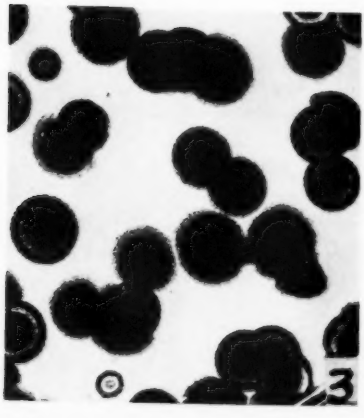
FIG. 5. Plaque formation of a suspension of MT_2L phage containing only one plaque type (natural size).

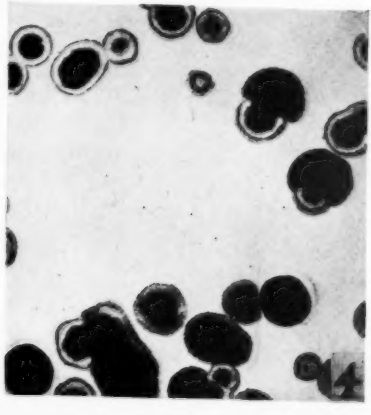
FIG. 6. Plaque formation of a suspension of $MT_{2a}L$ phage containing only one plaque type; 24 hr. incubation at 37° C. followed by alternate refrigeration and incubation at room temperature (24° C.) (natural size).

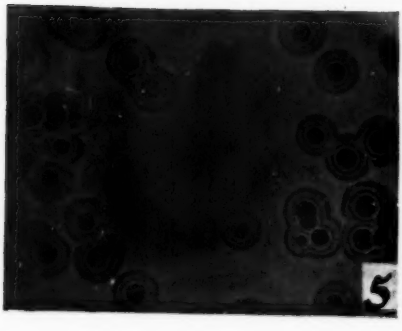
PLATE I











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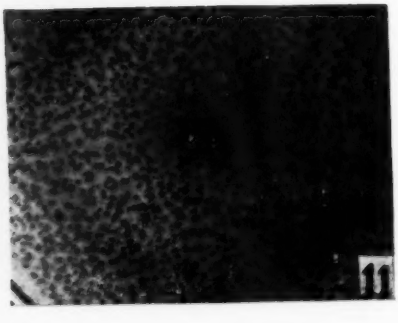
11

Characteristics of Phages

Plaque Type

The size of the plaques produced by the phages under the same conditions (pour plate method) remained fairly constant. Size in plaques ranged from pin point to about 4 mm. in diameter. Types of plaques varied from large halo plaques which produced concentric rings of lysis to small pin point plaques with no definite halo (Figs. 1-6, 10-11). Variations in plaque type of $MT_{2a}L$ and MT_2L phages (Figs. 3-4) were encountered during a study of plaque formation. At least three different types of plaques were observed when $MT_{2a}L$ phage was plated with its host. Single plaque suspensions of two such plaques investigated retained their character upon replating. The differentiation of these plaque types was based on the appearance of the halo.

According to size and type of plaques the phages found were classified into four groups (Table II). Groups three and four overlap slightly.

TABLE II

CLASSIFICATION OF 'ACID-FAST' PHAGES BY PLAQUE TYPE AND SIZE

Type and size of plaque produced	Phages	Group number
Large plaque 2-3.5 mm. in diameter which develops a halo after 48 hr. incubation at a lower temperature	MT_1	1
Large halo plaques 3-4 mm. in diameter which produce concentric rings of lysis about their plaques. The halos increase in size during alternate incubation and refrigeration	MT_2L, $MT_{2a}L$, MT_3, MS_4/607	2
Small haloless plaques about 1-1.5 mm. in diameter. Mutants of MT_2 and MT_{2a}	MT_{2s}, MT_{2as}	2a
Medium round plaques about 1-1.5 mm. in diameter which do not increase in size	MS_7, MS_4, MS_8, MS_1, M8016, MT_3/S_1	3
Small plaques, pin point to 1 mm. in diameter	MS_2, MS_3, MS_5, MS_6	4

FIG. 7. An electron micrograph of *M. smegmatis* phage, MS_7. The phage particles have two areas of density, one behind the other, with a central depression, as indicated by the shadow. Palladium shadowing. Magnification – 25,000 ×.

FIG. 8. Electron micrograph of same phage, MS_7, taken the following day. Slight variation between phage particles can be seen. Palladium shadowing. Magnification – 25,000 ×.

FIG. 9. Electron micrograph of *M. smegmatis* phage, MS_4. Palladium shadowing. Magnification – 25,000 ×.

FIG. 10. Plaques of *M. smegmatis* phage, MS_1, on avirulent *M. tuberculosis* ATCC 607; 24 hr. incubation at 37° C. followed by standing at room temperature (24° C.) (natural size).

FIG. 11. Plaques of same phage, MS_1, with 24 hr. incubation; 0.1 ml. of 10^{-5} dilution of phage was used (natural size).

Thermal Inactivation

A pure suspension of each phage was prepared and exposed to a specific temperature for 10 minutes after which it was tested against a susceptible host on solid agar. The above 'acid-fast' phages are inactivated at temperatures within the range 65°-85° C. They were found to fall into four groups (Table III). *M. phlei* phage MT_1 and MT_4 though isolated by slightly different methods were both inactivated at the same temperature. This further substantiates the belief that these two phages are alike.

TABLE III

THERMAL INACTIVATION OF 'ACID-FAST' BACTERIOPHAGES

Temperature range ° C. ± 1.5	Phages
65 – 70	MS_4/607, MT_3/S_1, MT_2L/Ph_2
70 – 75	MT_1, MT_4, MP_1
75 – 80	MT_2, MT_{2a}, MT_3, MS_1, MS_2, MS_3, MS_6, MS_7, MS_8, MS_9, ML_1, MR_1
80 – 85	MS_4, MS_5

Cross-resistance Tests

Cross-resistance tests have been employed by several authors (2, 4) as a means of differentiating between phages. The technique of Demerec and Fano (4) was adopted in this study. Resistant mutants were obtained for the strains Ph_1 and ATCC 607 by exposing surface streaked plates to MT_2, MT_{2a}, and MT_3 phages and propagating the growth from the lysed areas. Three to six bacterial mutants were tested against the phages by the usual techniques. The phages were classified into four groups. A close relationship was found to exist between this method and that of plaque type (Table IV).

Morphology

Mounts for electron photography for phages MS_4, MS_7 and MT_{2a} were prepared after a replica technique of Edwards and Wyckoff (6). The phages examined exhibited typical phage morphology (Table IV). Figs. 7, 8, illustrate two micrographs of phage MS_7 taken on two successive days. Slight variations in morphology are evident. Fig. 7 shows phage particles

TABLE IV

CLASSIFICATION OF 'ACID-FAST' BACTERIOPHAGES

Group No.	Plaque type	Cross-resistance tests	Serological cross-resistance tests	Activity	Morphology (in mu.)
1	MT_1	MT_1	MT_1	MT_1	MT_{28}
2	MT_2L, $MT_{28}L$, MT_2, $MS_4/607$	MT_2L, $MT_{28}L$, MT_2, MT_{28}, $MS_4/607$	MT_2L, $MT_{28}L$, MT_2, MT_{28}, $MS_4/607$	MT_2L, $MT_{28}L$, MT_2, MT_{28}, $MS_4/607$	Head dia. 115 Tail length 325 Tail width 15– 20
2A	MT_2, MT_{28}	Group 2	Group 2	Group 2	MS_4
3	MS_7, MS_4, MS_8, MS_1, M8016, MT_3/S_1	MS_4, MS_7, MS_8, M8016, MT_3/S_1	MS_1, MS_2, MS_6, MS_7, MS_8	MS_4, MS_7, MS_1	Head length 100–120 Head width 45– 50 Tail length 200–210 Tail width 13– 18
4	MS_2, MS_3, MS_5, MS_6	MS_1, MS_2, MS_3, MS_5, MS_6	MS_2, MS_4, MS_5	MS_3, MS_5	
5				MS_4, MS_6	MS_7
6				MS_2	Head length 120–165 Head width 55– 65 Tail length 200–225 Tail width 14– 18
7				M8016	
8				MT_3/S_1	

which have two areas of density, one behind the other, with a central depression, as indicated by the shadow. Phage MT_{2a} has a spherical head and a very long tail. In this respect it showed different morphological characteristics from the others.

It is expected that *M. smegmatis* phage will reveal a similar morphology but with a larger particle size because they produce small plaques and diffuse more slowly through 1% nutrient agar.

Serological Cross-resistance Tests

Sera were obtained against MT phages and MS_1 by injecting pure suspensions of phage ($0.4\text{-}3.6 \times 10^8$ phage particles per ml.) intravenously into rabbits. Five injections of increasing amounts from 0.5 to 2.5 ml. were made at five day intervals and the rabbits were bled from the ear seven days after the last injection. If the titer of the serum was unsatisfactory a booster dose was given. It was found that after the first course of injections the titer was satisfactory for use. The sera were stored in the refrigerator without preservative and were found to retain their activity unimpaired for months.

The sera were tested against homologous phages by mixing equal volumes of serum with phage so diluted in broth that upon assay of the final mixture about 100 plaques would be obtained in the absence of any neutralization. The mixtures were kept at 37° C. for one hour prior to plating by the pour plate technique (12). Reductions in plaque count to $\frac{1}{4}$ or less than that of control were noted as significant. In addition to homologous phages all the other phages were tested similarly to establish their serological grouping.

In every instance that has been investigated an antiserum neutralizes the homologous phage to a recognisably higher titer than any other phage. The phages fall into four different serological groups (Table V). MT phages and their mutants were found to belong to the same serological cross-resistance group. Such observations were recorded with *E. coli* phage and their mutants (11). Generally it can be concluded that there is good agreement between serological and cross-resistance tests.

Observations on Phage Typing of Mycobacteria

The strains examined included 50 saprophytes of which 13 were classified, one strain of BCG, and 144 pathogenic mycobacteria. The pathogenic group included 120 human, 14 bovine, and eight avian tubercle bacilli. This large

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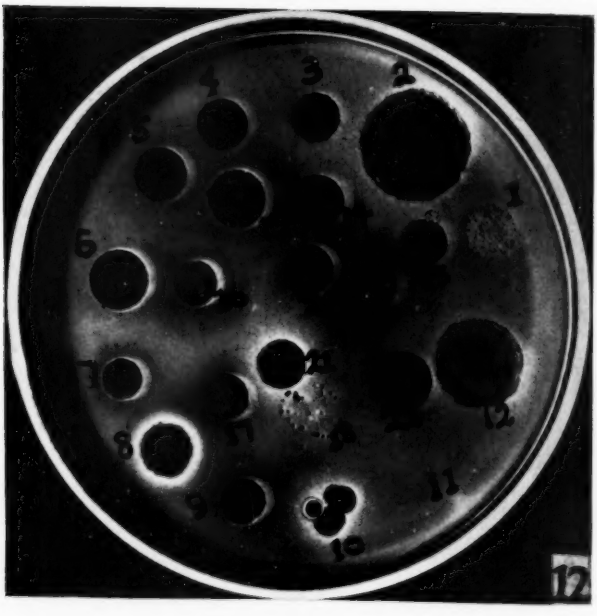
FIG. 12. Photograph illustrating the method used in testing mycobacteriophages against acid-fast microorganisms. The dark circular areas represent lysis of the strain *M. phlei*, Ph_1. The numbers correspond to the following phages. For clarification see Table I.

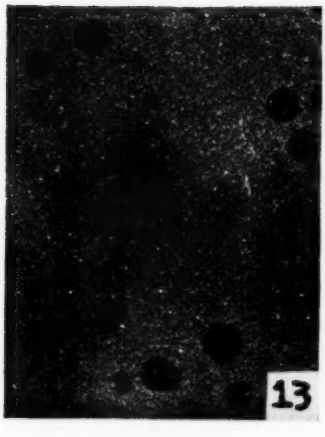
1.—MT_1 2.—MT_2 3.—MT_4 4.—MS_2 5.—MS_3 6.—MS_4 7.—MS_5
8.—MS_1 9.—MS_6 10.—MS_4/607 11.—MP_1 12.—MR_1 13.—ML_1
14.—MS_7 15.—MS_8 16.—MS_9 17.—MS_2 18.—M8016 19.—MS_3 20.—All
21.—MT_3/S_1 22.—MT_2L/Ph_2.

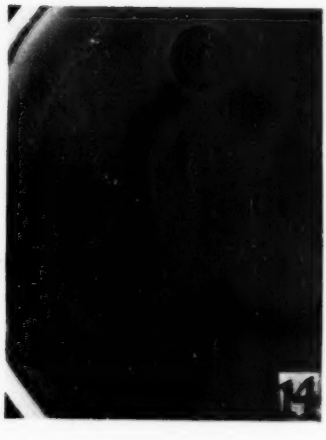
FIG. 13. Plaques of *M. phlei* phage, MT_1, on *M. phlei* strain, Ph_2.

FIG. 14. This photograph illustrates variation in plaque type of MT_3 phage. Phage was grown on ATCC 607 (natural size).

PLATE III







group of strains was selected in order to test as widely as possible the host-range pattern for all the phages isolated. The phages tested were undiluted stock phages. Twenty-two or 44% of saprophytic strains were lysed by one or more of the phages. Results were based on four tests done at intervals of three weeks. None of the phages showed activity for the pathogenic group. Nine or 24% of 37 unclassified saprophytic strains were lysed by one or more of the phages. Eight of these fell into a *M. smegmatis* pattern. One was lysed by phagus pellegrino. Results indicate a great deal of overlapping of the sensitivity pattern (Table V).

TABLE V

TYPES OF MYCOBACTERIA WITH RESPECT TO PHAGE PATTERN

Species and type		Phage pattern	No. of strains falling into pattern
M. smegmatis	A	MS_1-MS_9, ML_1, MR_1, MT_3/S_1	3
	B	MS_1-MS_5, MS_7-MS_9, MT_3/S_1	1
M. phlei	A	MT_1-MT_2, MS_1-MS_9, ML_1, MR_1, MT_3/S_1	1
	B	MT_1, MS_2, ML_1, MT_4	4
M. fribergensis	A	MS_1-MS_9, ML_1, MR_1	1
M. pellegrino	A	MP_1	1
M. rabinowitschi	A	MS_1-MS_7, MS_9, ML_1, MR_1	1
Avirulent *M. tuberculosis* ATCC 607	A	ML_1, MR_1, MT_3/S_1, MT_2-MT_3, MS_1-MS_9	1
Unclassified of *M. smegmatis* type		MS_1, MS_2, MS_4-MS_7	9
Total			22

The distribution of phage types among the species of mycobacteria is also illustrated in above table.

From range of activity and plaque type phages MS_3 and MS_9, and MS_2 and ML_1 appear to be alike. Phage MS_2 exhibits promising activity. It lysed 15 to 22 strains.

Discussion

The investigation of material from different sources for the presence of 'acid-fast' bacteriophages revealed that soil under the conditions of enrichment was a good source of phage. These results are in agreement with those of Gardner and Weiser (9) and Whittaker (20). However, several authors have isolated phages directly from soil filtrate (13, 10). In such an instance, the soil was generally shown to be rich in the microorganisms for which the phages were isolated. The finding that two samples of soil from the same source inoculated with the same strain can yield two different types of phage suggests that the nature of the soil and the size of the inoculum are the important factors in the success of phage isolation.

The failure of the tubercle bacilli to reveal any specific phage may possibly be attributed to the inability of these microorganisms to multiply in the soil and the failure of the existing phages to adapt themselves to strains of such a different nature. The addition of the medium to the soil most likely enhanced the growth of the soil microorganisms to such an extent that the conditions produced were detrimental to phage reproduction. However, many samples of soil should be investigated before negative results be accepted.

The method of bulking cultures of 'acid-fast' strains and testing the resultant mixture for bacteriophages was held in preference to classical methods in that it is likely to yield a larger number of bacteriophages. Such was not the finding. However, it must be borne in mind that some bacteriophages when present in the same environment can interfere with the development of others (3) and thus perhaps not all the phages present in a mixture of a number of strains will be propagated. This method has been used with success by Warner (18). It must also be borne in mind that in testing pathogenic strains for a lysogenic culture the increased incubation period so necessary for their growth can become inhibitive to phage reproduction.

Indirect evidence obtained suggests that possibly less than 5% of the acid-fast microorganisms tested were phage carriers. This percentage is low for a phenomenon that is considered to be exceedingly common with other genera. The findings of Bordet and Bordet (1) that an optimum concentration of ionic calcium is necessary for the clearing of broth cultures of *Shigella shiga* by phage may indicate that medium which lacks ionic calcium in desired amounts is one of the factors why bacteriophages cannot be isolated from lysogenic cultures. Furthermore, the appearance of plaques on some tubercle bacilli and the fact that phage is adsorbed onto pathogenic cells without subsequent lysis indicates that possibly cofactors are needed for phage penetration and multiplication. This observation needs further investigation. Though adsorption takes place the factors that block subsequent growth of phage which normally would lead to lysis also seem to interfere with the metabolism of the bacterium. This was observed when H37rv was grown in the presence of a relatively high concentration of phage MS_7 or MT_3. A similar culture containing no phage grew much more rapidly. Associations of

phage–host comparable to ones observed here have been reported previously (14, 7, 15, 19).

The characteristics of bacteriophages described earlier are typical of other phages. Of interest is the phenomenon of plaque formation and variation of plaque types of MT phages. The good keeping qualities of the 11 bacteriophages and their range of activity suggests that they could be applied to bacteriophage typing of the mycobacteria (Table V). The typing scheme presented is very tentative and of limited nature. New host range mutants or specific phages will have to be developed for both the pathogenic and nonpathogenic group. As this is achieved the number of phages within the species will increase, making the scheme more cumbersome. However, the development of a few bacteriophages with a wide range of activity for each species and the use of these in their critical test dilution would decrease the number of phages used and improve upon the technique. This point of view is now being further investigated.

Acknowledgments

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FURTHER STUDIES ON LIPOTROPISM IN THE DOMESTIC DUCK (White Pekin)[1]

By Denyse D. Duchesne and Richard Bernard

Abstract

On choline-free diets containing 10, 18, and 28% casein, the total liver lipids of ducklings were found to be 34.8, 25.0, and 13.8% respectively. Supplementation of the 10% casein diet with 0.5% methionine increased the liver lipids. The same amino acid has no effect at the intermediate level of casein while it is lipotropic at the 28% casein level. In similar experiments with a purified soybean protein (low in methionine) fed at levels of 12, 21.7, and 27.9%, the liver lipids were 31.8, 26.6, and 27.6% respectively. These values do not differ significantly from each other. Methionine was found to be lipotropic at any of these three levels of soybean protein, and more so as the level of the soybean protein increased in the diet. From these results, it is concluded that the purified soybean protein used is a nonlipotropic protein, while casein is definitely lipotropic in ducklings. The lipotropic activity of methionine is closely related to the quality and quantity of the protein in the diet. On high fat diets containing 22, 32, and 42% fat, choline had no lipotropic effect. The average liver lipids for all groups ranged from 11 to 15%. High fat diets are less inducive of fatty livers than high carbohydrate diets. Ducklings fed a fat-free, choline-free basal diet presented livers with an average total lipids of 18%. Supplementing the basal diet with 1% cholesterol increased the liver lipids to 22%. Inositol is lipotropic either in the presence or absence of cholesterol, but choline was much more effective under similar conditions. The two lipotropic factors have a synergistic action and the reduction of the liver lipids is primarily on the glyceride fraction. Cholesteryl ester was at its lowest value in the group fed the basal diet supplemented with inositol only.

Introduction

The present work has been undertaken in order to get additional information on lipotropism in the domestic duck (White Pekin). In a previous study, Bernard and Demers (1) noted that supplementation of low-casein diets with methionine increased slightly the liver lipids of ducklings. Later on, the same authors (10) fed this amino acid along with a 28% purified soybean protein, and a definite lipotropic effect was observed. It was thus suspected that the lipotropic action of methionine is related to the level of protein in the diet. Consequently, this aspect was further studied and the results are presented in the first two series of experiments reported in this paper.

In a third series of experiments, the lipotropic activity of choline was investigated with diets containing 22% fat or more. In our previous work, diets containing only 12% fat had been used.

Finally, a fourth series of experiments dealt with the comparison of the lipotropic action of inositol and choline in animals fed fat-free diets with and without cholesterol.

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Contribution from the Department of Biology, Laval University, Quebec, and the Quebec Zoological Garden, Charlesbourg, Quebec, with financial assistance from the National Research Council of Canada.

Holder of a scholarship from the Scientific Research Bureau, Province of Quebec.

Material and Methods

(a) *Care and Housing of Animals*

Day-old white Pekin ducklings were used in these studies. Upon receipt from the hatchery, they were placed in heated batteries equipped with raised-screen bottoms. Ten ducklings were located in each compartment measuring 3 ft. square. The birds used in the first three experiments were fed a commercial duck feed for two to three days prior to the experimental period. Those used in the last experiment involving fat-free diets were kept six days on the natural diet. Food and water were allowed ad libitum. Each group consisted of 10 animals weighed three times a week, and the experimental periods lasted from 16 to 20 days.

(b) *Analytical Procedures*

At the end of each experiment, the birds were killed by decapitation, their livers weighed, and samples taken for analyses. In the first three experiments, duplicate liver samples were analyzed for total lipids, according to a modified procedure of the method of Shipley *et al.* (16). The modification consisted in evaporating most of the solvents on a water bath following up with a final evaporation in a vacuum oven held at 40° C. In the fourth experiment, the lipids were extracted from the pooled livers of each group, according to the procedure described by Best *et al.* (5). In this procedure, the lipids are finally dissolved in petroleum ether. Aliquots of this solution are taken for determinations of total lipids, free and total cholesterol, and phospholipids. A modified procedure (17) of the Schoenheimer–Sperry method was used to determine the free and total cholesterol. Total phosphorus was estimated by the method of Fiske and Subbarow as presented in Koch and Hanke (13). Phospholipids were calculated by multiplying the phosphorus content by the factor 25.2 while the content of bound cholesterol multiplied by 1.68 gave the weight of cholesteryl esters; the undetermined residue, calculated by difference, is expressed as glyceride.

(c) *Description of Diets*

The composition of the basal diets is given in Table I. Basal diets R-83 and R-89 are low-protein diets which were used to study the lipotropic effects of methionine in the presence of different levels of casein or purified soybean protein. Since the purified soybean protein contains only 83% crude protein (14), the percentage was raised accordingly (except for groups 11 and 12, Table III) in order to permit comparison with the casein diets. Basal diet R-102 contains 22% fat and was devised to study the lipotropic activity of choline in ducklings fed high fat diets. The protein moiety of this diet consisted of 28% soybean protein, a protein low in methionine (14). Finally, basal diet R-108 is fat-free and resembles that used by Best *et al.* (6). It was used primarily to evaluate the lipotropic activity of inositol. According to these authors, a demonstration of the lipotropic effect of inositol can only be

shown when it is added to a hypolipotropic diet devoid of fat. The vitamins were incorporated to the diet in the following way: alpha tocopherol was dissolved in the oil fraction, while biotin, pteroylglutamic acid, the vitamin A and D_3 concentrate were distributed as solutions over the protein component of the diet and thoroughly mixed. All other vitamins listed were premixed with a certain amount of casein or soybean protein so that 1 gm. of the mixture contained the vitamins required for 100 gm. of the diet.

TABLE I

COMPOSITION OF BASAL DIETS

Ingredients	Basal diet number			
	R-83	R-89	R-102	R-108
	Composition			
	%			
Main ingredients				
Sucrose	65	63	37	72
Casein, Labco	10	—	—	8
Soybean protein*	—	12	28	—
Gelatin	—	—	—	12
Corn oil	10	10	10	—
Crisco†††	—	—	10	—
Fish oil blend**	2	2	2	—
Liver fraction L***	4	4	4	—
Salts IV†	5	5	5	5
$CaH_4(PO_4)_2 \cdot 2H_2O$	1	1	1	—
Ruffex	3	3	3	3
	Mgm. per 100 gm. of diet			
Vitamins				
Thiamine hydrochloride	0.4	0.4	0.4	0.4
Riboflavine	0.8	0.8	0.8	0.8
Calcium pantothenate	2.5	2.5	2.5	2.5
Nicotinic acid	4.0	4.0	4.0	4.0
Pyridoxine hydrochloride	0.4	0.4	0.4	0.4
Biotin	0.02	0.02	0.02	0.02
2-Methyl naphthoquinone	0.1	0.1	0.1	0.1
Pteroylglutamic acid	—	—	—	0.1
p-Aminobenzoic acid	2.0	2.0	2.0	2.0
α-Tocopherol	10.0	10.0	10.0	—
Inositol	100.0	100.0	100.0	—
Vitamin A and D_3 concentrate††	—	—	—	16.0

* *"Alpha protein". The Glidden Company, Chicago, Ill.*

** *Vadol Type C, 2400 A, 400 D (A.O.A.C. chick units). Blended fish oils fortified with vitamin D_3. Ayerst, McKenna, and Harrison, Montreal, Que.*

*** *A source of the unknown vitamins of the B-complex. Wilson Laboratories, Chicago, Ill.*

† *Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B. J. Biol. Chem. 138 : 459. 1941.*

†† *Special concentrate containing 1,000,000 A and 50,000 D_3 per gram. Prepared by Ayerst, McKenna, and Harrison, Montreal, Que.*

††† *Hydrogenated cottonseed oil.*

Results and Discussion

A. *Influence of Different Levels of Casein or Soybean on the Lipotropic Activity of Methionine*

The results of the first two series of experiments are presented in Tables II and III. It will be seen that the liver lipids of both control groups is very high, 34.8 and 31.8% for groups 1 and 7 respectively. Increasing only the casein content of the ration did lower significantly the liver lipids. Thus, ducklings fed a 28% casein diet (group 5) presented an average of 13.8% liver lipids as compared with 34.8% for the control group fed a diet containing 10% casein. The data given in Table II thus establish clearly the lipotropic activity of casein in this avian species. In mammals, particularly the rat, such a fact has been shown in 1935 by Best and Huntsman (4) and by Channon and Wilkinson (9). It would be interesting to find out whether by increasing further the casein content, one could have normal values for liver lipids in ducklings. In rats, Best *et al.* (2) have found that a 30% casein diet is sufficient to obtain normal liver fatty acids. It appears however that, for a given level in the diet, casein is less lipotropic in ducklings than in rats. This difference is probably related to methionine availability in the digestive tract of the animal species considered.

On the other hand, when the percentage of soybean protein is increased up to 27.9% (Table III), there is no significant decrease in the liver lipids. Thus, it appears that soybean protein is nonlipotropic. No doubt this lack of lipotropic activity is to be related to its low methionine content which, according to Kratzer *et al.* (14), amounts to 1.3%, while casein contains 3.5% methionine (8).

If we now examine the lipotropic activity of methionine in relation to the different levels of proteins, it will be seen that this amino acid was most effective when supplementing the soybean protein diets. Such results are in agreement with the fact that this protein is deficient in methionine. With the casein diets, the results are quite different. In fact, supplementing the 10% casein basal diet with 0.5% methionine (group 2) increased significantly the liver lipids. A similar observation has been already reported in our previous work (1) where a 9% casein diet had been used. It is probable that the augmentation in liver lipids in the above instances is the result of some amino acid imbalance. It is interesting to note that, at the 18% casein level (Table II, group 4), methionine did not alter the liver lipids picture. On the other hand, at the level of 28% casein, methionine lowered the average liver fat from 13.8 to 7.4% (groups 5 and 6), although this difference is not quite significant ($t = 2.0$). The lipotropic role of the casein component of the diet stands out more clearly when groups 4 and 6 (Table II) are compared. Here increasing the percentage of casein from 18 to 28% in the presence of 0.5% methionine lowered the average liver fat from 23.8 to 7.4%. One may question whether this lipotropic effect is due only to the increased total methionine of the diet or to a beneficial effect of the other amino acids of the

TABLE II

LIPOTROPIC ACTIVITY OF METHIONINE AT DIFFERENT LEVELS OF CASEIN

Group No.	Supplement to the 10% casein basal diet R-83	Total liver lipids, % wet weight	t*	Birds with perosis	Average weight, gm.	
				Birds on experiment	4 days	24 days
1	None	34.8 ± 3.1**	—	1/10	84.3	162 ± 13.1
2	DL-Methionine 0.5%	47.9 ± 2.3	3.4	2/10	85.0	189.2 ± 12.9
3	Casein 8%	25.0 ± 2.7	2.4	7/10	76.5	280.5 ± 9.0
4	Casein 8% DL-Methionine 0.5%	23.8 ± 4.0	—	6/10	76.3	232.9 ± 8.8
5	Casein 18%	13.8 ± 3.2	4.7	9/9	68.4	251.8 ± 19.7
6	Casein 18% DL-Methionine 0.5%	7.4 ± 0.6	—	7/8	68.6	247.1 ± 18.5

* $t > 2.3$ significant by comparison with control group.

** *Standard error* $= \sqrt{\left(\frac{\Sigma d^2}{n(n-1)}\right)}$.

Comparison of liver lipids: groups 2 and 4, $t = 5.2$
groups 4 and 6, $t = 3.9$
groups 5 and 6, $t = 2.0$
groups 3 and 5, $t = 2.7$.

TABLE III

LIPOTROPIC ACTIVITY OF METHIONINE AT DIFFERENT LEVELS OF SOYBEAN PROTEIN

Group No.	Supplement to the 12% soybean protein basal diet R-89	Total liver lipids, % wet weight	*t*	Birds with perosis	Average weight, gm.	
				Birds on experiment	5 days	21 days
7	None	31.8 ± 3.3	—	2/10	95.2	160.6 ± 5.7
8	DL-Methionine 0.5%	23.8 ± 2.2	2.1	7/10	94.2	325.5 ± 19.4
9	Soybean protein 9.7%	26.6 ± 3.1	1.2	9/10	86.5	251.3 ± 14.4
10	Soybean protein 9.7% DL-Methionine 0.5%	16.5 ± 1.7	—	9/10	86.9	332.8 ± 20.2
11	Soybean protein 15.9%	27.6 ± 2.0	1.1	10/10	78.6	228.1 ± 14.3
12	Soybean protein 15.9% DL-Methionine 0.5%	9.9 ± 1.1	—	9/10	78.3	278.3 ± 14.7

Note: Comparison of liver lipids: groups 8 and 10, t = 2.8
groups 8 and 12, t = 6.1
groups 10 and 12, t = 3.3
groups 9 and 10, t = 2.9
groups 11 and 12, t = 7.7.

casein. As a matter of fact, Elvehjem (12) has reported recently that, in the rat, amino acids such as proline, threonine, and glycine function in addition to methionine in preventing the deposition of fat. Furthermore, Dick *et al.* (11) have noted the accumulation of fat in the liver of the same species during the course of deficiencies of threonine or of lysine.

Except for groups 1, 2, and 7 where growth was at its lowest value, the incidence of perosis was very high. This may indicate that whatever amount of choline there was available through synthesis from methionine, it was all used up primarily for lipotropic action and that none was left for the prevention of perosis or for growth which at its best did not exceed half the normal. This was expected, for all diets were free of choline, a vitamin essential for growth of ducklings. Increasing the protein content of the diets, whether it was casein or soybean protein, generally resulted in a slightly improved growth. Supplementation with methionine improved growth of the animals fed the soybean diets (Table III), while it did not in the groups receiving the casein diets (Table II).

B. Lipotropic Action of Choline in the Presence of Different Levels of Fat in the Diet

In our previous studies (1, 10) on lipotropism in ducklings, it was shown that choline lowered the liver lipids of ducklings fed diets containing 12% fat. These preliminary studies were extended, starting with a basal diet containing 28% soybean protein and 22% fat. The lipid fraction of basal diet R-102 consisted of 10% corn oil (Mazola), 10% Crisco, and 2% fish oil blend. The fat percentage of the basal diet was increased to 32 and 42% by the addition of Crisco and the sucrose decreased accordingly. The results of these experiments are presented in Table IV.

The first striking result in this series of experiments is the absence of lipotropic activity of choline at these high levels of fat in the diet. The livers were moderately fat on all diets, with values ranging from 11.0 to 15.8%. It may be that the absorption of choline in the intestine is prevented by fat. It is also possible that the ducklings are not physiologically or anatomically equipped to handle high fat diets and that the mere addition of choline does not remedy the situation. The natural diet of ducklings whether in the wild or domesticated is low in fat and high in carbohydrate. The albino rat, on the contrary, handles high fat diets readily. In the early work of Best and Huntsman (3) on lipotropic factors in rats, diets containing 40% fat were used to produce fatty livers, and choline was found to exert a marked lipotropic action in this species.

In view of these negative results, it should prove useful to investigate further the lipotropic activity of choline with diets containing levels of fat varying from 12 to 22% fat.

The results of these experiments also show that the production of fatty livers in ducklings does not necessitate a high fat diet but rather a diet low in protein with medium fat content, i.e., about 12%. In Table IV are also

TABLE IV

LIPOTROPIC ACTION OF CHOLINE IN PRESENCE OF HIGH FAT DIETS

Group No.	Supplement to the 22% fat basal diet R-102	Total liver lipids, % wet weight	Birds with perosis	Average weight, gm.		t
			Birds on experiment	4 days	23 days	
13	None	13.6 ± 1.8	6/10	72.4	208.6 ± 24.1	
						5.2
14	Choline chloride 0.3%	14.2 ± 1.5	0/10	70.9	385.1 ± 25.7	
15	Crisco 10%	13.8 ± 1.5	4/10	75.1	241.1 ± 24.3	
						5.1
16	Crisco 10% Choline chloride 0.3%	15.8 ± 2.8	0/10	75.5	410.6 ± 23.9	
17	Crisco 20%	13.2 ± 1.9	7/10	79.0	235.8 ± 19.9	
						0.2
18	Crisco 20% Choline chloride 0.3%	11.0 ± 2.4	0/10	79.0	225.0 ± 40.9	

Note: Comparison of weights at 23 days: groups 14 and 16, $t = 0.8$
groups 14 and 18, $t = 3.3$
groups 16 and 18, $t = 3.9$.

included observations on perosis and growth. As will be noticed, perosis was absent in those groups receiving choline. This vitamin also improved growth markedly except in group 18 which received the 42% fat diet. The poor growth of this latter group may be related to the nature of the diet which was very greasy and may have caused poor food and water consumption.

C. The Lipotropic Action of Choline and Inositol in Ducklings Fed Fat-free Diets With and Without Cholesterol

The results of this third series of experiments are presented in Table V. The data refer to pooled livers for each group and consequently did not permit statistical analysis. The analytical results for total lipids, phospholipids, cholesterol (free, total, and esterified), and glyceride are presented on a fat-free dry residue basis. For comparison with the preceding experiments, the total lipids are also given on a wet weight basis.

It will be recalled that the use of a fat-free basal diet (R-108) was prompted by the fact that different kinds of fat may abolish the lipotropic effect of inositol. This observation was first made by Beveridge (7) and confirmed in more elaborate studies by Best *et al.* (6).

If we first consider the total lipids (wet basis), it will be seen that in control group (group 19), the livers contained 18.4% lipids, which is about three times the normal value. Thus, it is possible in ducklings to produce fatty livers with fat-free diets. The livers of group 22 which received a supplement of 1% cholesterol contained a slightly higher amount of fat of 21.9%. It appears that cholesterol may not induce fatty livers in ducklings to any great extent. In similar experiments with rats, Ridout *et al.* (15) have reported values of the order of 27 to 39%. It is probable that, for a given hypolipotropic diet, fatty livers are induced more easily in rats than in ducklings.

Choline again was very effective in reducing the liver fat in groups 21, 24, and 25, with total lipids values of 7.3, 6.1, and 4.6% respectively. Choline was equally active whether cholesterol was present or absent in the diet. The lowest liver lipid value of 4.6% (group 25) was observed when choline and inositol were added to a 1% cholesterol diet. This points to a synergistic effect of these two factors. Such a fact has already been observed in the rat by Ridout *et al.* (15).

If we now pass to inositol (groups 20 and 23), it will be seen that this substance is definitely lipotropic whether in the presence or absence of cholesterol. It is however so to a much less extent than choline.

Both lipotropic factors exert their effect primarily on the glyceride fraction of the livers. A pronounced reduction of cholesteryl esters was noted in one instance only (group 20), which received a supplement of 0.3% inositol. Such a reduction, however, was not observed on cholesterol fed animals. Choline exerted a slight lowering of free cholesterol in group 24 which received a 1% cholesterol supplement. Apparently, the addition of 1% cholesterol to the basal diet increased the total lipids through the glyceride fraction rather than the cholesterol fractions.

TABLE V

LIPOTROPIC EFFECT OF CHOLINE AND INOSITOL WITH AND WITHOUT CHOLESTEROL

(Average initial weight of ducklings 187 gm. (range 168-210 gm.)). (Duration of experiments 19 days)

Group No.	Supplement to the fat-free basal diet R-108	Survivors / Starters	Weight increase, gm.	Liver			Composition of liver lipids % fat-free, dry residue weight					
				Average wet weight, gm.	Average fat-free dry residue weight, gm.	Total liver lipids, % wet weight	Total lipids	Phospho-lipids*	Cholesterol			Glycerides†
									Free	Total	Ester**	
19	None	10/10	176	22.7 2.1	3.8	18.4	110.3	5.27	1.33	2.04	1.19	102.5
20	Inositol 0.3%	8/10	90	13.2 1.2	2.5	10.5	55.4	10.56	1.21	1.50	0.49	43.1
21	Choline chloride 0.3%	10/10	177	18.6 2.6	3.9	7.3	35.0	9.93	1.37	2.08	1.18	22.5
22	Cholesterol 1%	8/10	118	19.6 3.1	3.2	21.9	132.2	9 69	1.35	2.08	1.24	119.9
23	Cholesterol 1% Inositol 0.3%	9/10	199	20.4 2.7	4.3	15.2	81.8	1.24	1.23	2.02	1.33	78.0
24	Cholesterol 1% Choline chloride 0.3%	9/10	199	16.7 1.9	3.7	6.1	27.4	6.53	1.07	1.76	1.16	18.6
25	Cholesterol 1% Inositol 0.3% Choline chloride 0.3%	9/10	286	19.7 1.4	4.4	4.6	20.8	1.60	1.54	2.24	1.16	16.5

* *Phosphorus content* × *25.2.*
** *Bound cholesterol* × *1.68.*
† *Total lipids—(phospholipds + free cholesterol + bound cholesterol).*

The interpretation of the phospholipids data is somewhat difficult. We are at a loss to find a reason for the extremely low values for groups 23 and 25. In both groups, cholesterol and inositol were present in the diets.

Though the weight changes of the ducklings during the 19-day experimental period are not of prime interest in this study, it must be noticed that inositol or cholesterol (groups 20 and 22) depressed growth significantly. A combination of cholesterol with inositol or choline improved growth only slightly. It is surprising to note that choline alone (group 21) was not effective as a growth factor when fed along with a fat-free diet. A strong growth response was noticed only in group 25 which received simultaneous addition of choline, inositol, and cholesterol. The survival rate was high in all groups. No typical perosis was noticed though many birds exhibited unsteady gait.

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PURIFICATION OF DIPHTHERIA TOXOID BY CONTINUOUS ELECTROPHORESIS ON FILTER PAPER[1]

By M. D. Poulik

Abstract

A separation of purified diphtheria toxoid in a continuous electrophoresis apparatus has been achieved and resulting components have been collected in quantities sufficient for testing and elaboration. The simple and inexpensive apparatus constructed of materials readily available is described and some factors concerned with the separation of the components are discussed. The results of the biological tests carried out on the components are outlined.

Introduction

There are numerous reports on the electrophoretic analyses of complex protein mixtures following the method and utilizing the apparatus devised by Tiselius (22). The description of continuous electrophoresis, however, is restricted to a very few publications.

There is no apparatus comparable to the classical model with respect to sharpness of separation and quantitative accuracy. Its disadvantages, however, lie not only in the high cost of the equipment and its maintenance but also in the difficulty in applying it when relatively large quantities of the separated fractions are desired for investigation. Continuous electrophoresis provides a convenient means of separating components in relatively large quantities.

In principle, continuous electrophoresis makes use of a porous supporting medium through which a steady, continuous flow of a buffer is maintained. The material to be investigated is introduced into the moving buffer and an electrical field is established perpendicular to the flow. If the material contains molecules which differ in electrical charges (proteins, amino acids, enzymes, etc.), these molecules will move with different mobilities in the electrical field. The position of the molecules in the porous medium at any moment will be the resultant of their electrophoretic mobilities and the speed of flow of the buffer. Therefore, molecules differing in electrical charges will also differ in final position and separation will result.

The design of a practical apparatus for this purpose is beset by many technical difficulties such as maintenance of a uniform electrical field, reduction of evaporation, and adjustment of rate of flow. When components differing only slightly in electrophoretic mobilities are present the difficulties are correspondingly greater.

From a consideration of the principle involved it is evident that the porous medium is one of the most important factors governing successful separation. Svensson and Brattsen (20) used glass powder packed in a Lucite box.

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Grassmann and Hannig (7) reported the first successful continuous electrophoresis of amino acids and proteins in filter paper. Durrum (4) also used filter paper for the separation of dyes, amino acids (9 in a mixture), and serum proteins.

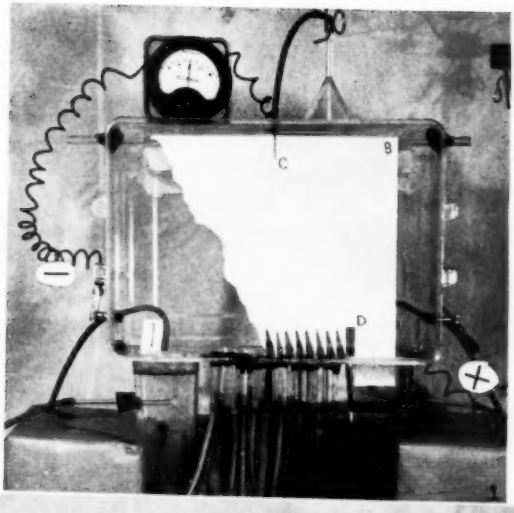
Since heat is produced by the applied current different arrangements have been devised for cooling the system and for preventing undue evaporation from the buffer. If glass powder or sea sand is used as the porous medium, the cooling is easily achieved by circulating water around the box in which these materials are enclosed. With filter paper as the porous medium, three arrangements have been devised to suppress evaporation of the buffer: (1) enclosing the filter paper in a moist chamber (Durrum (3, 4), Grassmann, Hannig, and Knedel (9)); (2) clamping the filter paper between two glass plates and cooling by immersing the plates in a bath of an immiscible substance such as chlorobenzene (Cremer and Tiselius (2), Schneider (19)); (3) clamping the filter paper between glass plates treated with silicone grease to prevent adhesion and performing electrophoresis either in a cold room (Kunkel and Tiselius (11)) or in a bath of circulating cold water (Brattsen and Nilsson (1)).

The investigation outlined in this report applied the moist chamber method. This was chosen because of its simplicity and the sharpness of separation achieved (4). In previous work (17) purified diphtheria toxoid was subjected to simple (i.e. noncontinuous) electrophoresis on filter paper. A separation into three components was achieved. Flocculation tests, performed in an agar layer, showed the flocculating activity to be localized in one only of the three fractions. The volumes of material which could be separated by simple electrophoresis were too small to permit adequate studies of the separated fractions and accordingly an apparatus for continuous electrophoresis on filter paper was constructed. The apparatus employed is a modification of that of Durrum (4) and is described below in this report. Because of the simplicity and low cost of construction it is hoped that this contribution to the field of continuous electrophoresis will prove helpful for further development.

Part I. Apparatus

Description of the Apparatus

The apparatus, Figs. 1 and 2, consists of a moist chamber in the form of a commercial plastic 'refrigerator box' (32 cm. × 11 cm. × 24 cm.). A filter paper curtain is shown as a sheet which has been torn to expose structures behind it. The curtain dips into buffer contained in a plastic trough cemented to the back wall of the box. The filter paper draws a steady supply of buffer from the reservoir trough which is filled through a rubber tubing with attached funnel. To prevent evaporation this rubber tubing is closed with a clamp when the apparatus is in operation. A glass rod, affixed to either side of the box a little above and in front of the trough, holds the curtain in position. The base of the box is drilled with 14 holes, each fitted with a short glass tube which is fixed in position by plasticine. The tubes are arranged in two rows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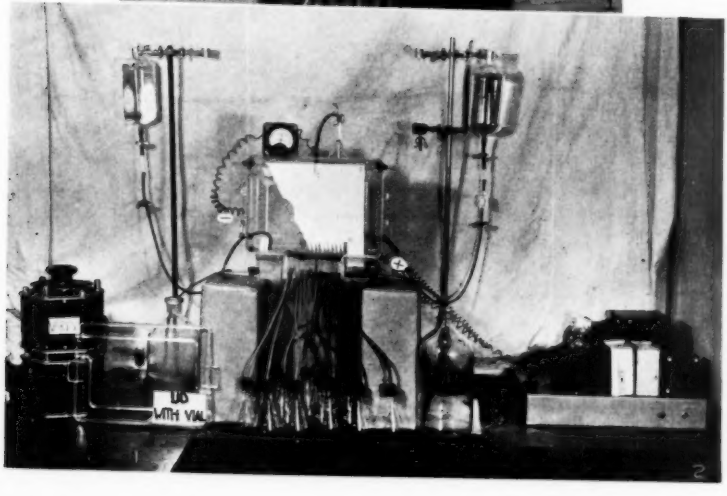
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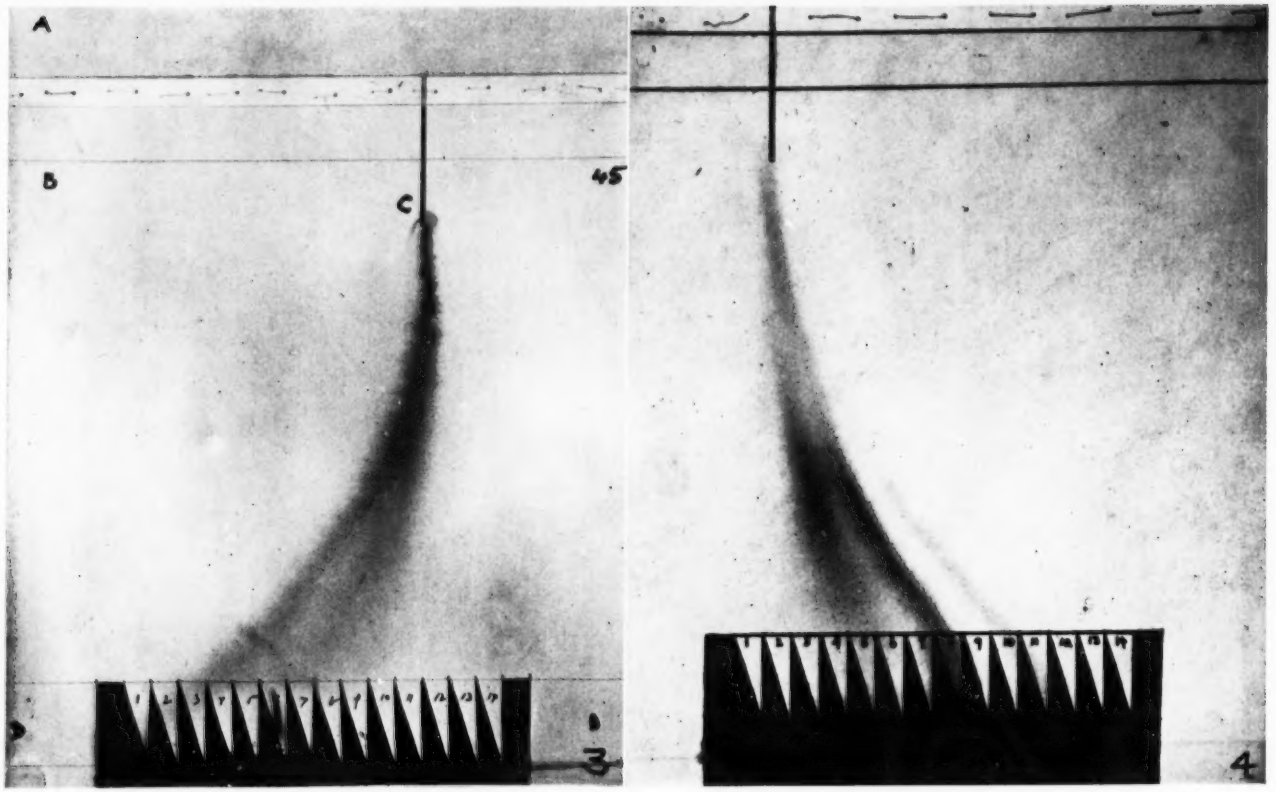

PLATE II

in a staggered fashion to accommodate the 14 drip-points cut from the bottom of the filter paper curtain. The glass tubes lead through rubber tubing to 14 Erlenmeyer flasks (125 ml.) which serve as collecting vessels; samples can then be taken during the progress of the experiment without opening the moist chamber. By clamping the rubber tubing leading to the flask, the equilibrium established in the chamber will not be disturbed.

The sides (*D*) of the curtain are somewhat longer than the rest of the face and protrude through slits (1 cm. × 5 cm.) cut in the base of the box to dip into the electrode comparments. These compartments, also consisting of standard plastic refrigerator food storage boxes (7.5 cm. × 5.5 cm. × 5.5 cm.) are cemented in place with acetone. They hold the platinum electrodes (gauge 30) which have their leads fixed to the side walls so as not to interfere with their automatic washing system. The electrodes are bathed by buffer passing through rubber tubing (1, Fig. 1) which enters the box through the side walls and enters the electrode compartments through holes drilled in the base of the box. The tubing (2, Fig. 2) leads from large plasma bottles which serve as reservoirs for the washing-buffer. To maintain a constant level of the buffer in the electrode compartments, glass tubes bent to form a right angle are seen protruding through rubber stoppers in the side walls of the compartments. Superfluous buffer is thus drained off via rubber tubing into large bottles. This arrangement provides for a continuous washing of the electrodes and maintains the buffer in the electrode compartments at the same pH as the buffer flowing down the curtain.

A vial, filled with the solution to be separated, is fastened to the inner surface of the lid of the box (Fig. 2). A narrow wick, but from the filter paper curtain but still connected with it (Fig. 1, *C*) is introduced into the vial and the box closed by the lid. The material is drawn from the vial through the wick into the filter paper curtain and is carried downwards by the steady gravitational flow of the buffer in the curtain. Hence only a current needs to be established in order to carry out an electrophoresis in the second direction. A standard power supply (15) was used in most experiments. However, a full-wave rectifier with a condenser-choke filter of our own design capable of functioning at voltage as high as 1700 volts, was employed in a later stage of the work.

Experimental

Filter Paper

Three types of filter paper were tested: Schleicher and Schull No. 598, Munktell No. 20 (Sweden), and Whatman No. 1. The paper was cut as in Fig. 3 (*B*) and a strip (*A*) of Whatman 1 (24 cm. × 5 cm.) was sewn to the top edge of the curtain. This strip served to regulate the flow of buffer from the trough so that the solution would not be drawn too quickly into the curtain. A wick 5.5 cm. long and 0.8–1.0 mm. wide was cut from the top of the curtain at *C*.

Buffers

The two buffers used were veronal (barbituric acid – sodium barbiturate) and Michaelis (13). Veronal buffer of pH 8.55 and ionic strength 0.1 was prepared by dissolving 20.62 gm. sodium barbiturate and 3.69 gm. barbituric acid in 2 liters of distilled water. Michaelis buffer (13) of pH 6.10 was prepared by mixing 200 ml. stock solution (19.428 gm. sodium acetate and 29.428 gm. sodium barbital in 1 liter of distilled water) with 280 ml. *N*/10 hydrochloric acid to make 1 liter with distilled water. The 8.5% sodium chloride, as given in the original description of this buffer, was omitted and the ionic strength thus lowered. Each kind of filter paper was tested with the two buffers under the following conditions. The current was tested over a range from 1–10 ma., the voltage from 80–500 v., the rate of flow of the buffer from 0.5–4 hr., as measured from top to bottom of the curtain, and duration of the experiment from 3–70 hr. The width of the wick was varied from 0.5–1.5 mm.

Results

Diphtheria toxoid was subjected to continuous electrophoresis in the apparatus described and considerable quantities of the separated fractions were collected. By using veronal buffer at pH 8.55 and ionic strength 0.1 with an applied current of 7 ma. at 350 v., two components were separated and subsequently differentiated (Fig. 3). With Michaelis buffer at pH 6.1 and an applied current of 8 ma. at a voltage of 250 volts, a separation into three components was achieved (Fig. 4).

Discussion

The discussion will be limited to stating the experience gained through the use of the apparatus in the separation of diphtheria toxoid. The electrophoretic mobilities of the two nonflocculating components differed very little. More than 60 separate experimental runs under varying conditions were made to determine the optimum for satisfactory separation. Once established, constant results were readily obtainable. The thickness and kind of the filter paper determined the rate of flow of the buffer in the curtain. At a satisfactory rate of flow the buffer reached the bottom of the curtain in 1.5 to 2 hr. Whatman No. 1 fulfilled this requirement but even low currents caused overdistillation and deposition of salts of the buffer on the paper. Schleicher and Schull 598 paper as well as Munktell 20 allowed the buffer to reach the bottom of the curtain in a half and three-quarters of an hour respectively which was too rapid for satisfactory separation. The addition of glycerol to the buffer did not improve the results. However, the substitution of 5–15% polyvinylpyrrolidone (PVP) slowed the flow to three hours, but darkening of the filter paper, especially in the vicinity of the electrodes, resulted and the risk of fire was always imminent. Schleicher and Schull 598 paper was found to give sharper separation under all explored conditions, when compared with Whatman No. 1 or Munktell 20. The final solution of this problem is

described under "Experimental: Filter Paper". The addition of 50 mgm. of PVP to each ml. of toxoid allowed the 1 mm. wick to introduce the right amount of toxoid into the curtain. In order to maintain a constant pH of the buffer flowing in the paper curtain, the electrodes are washed with buffer of the same ionic strength at a rate of 100-150 ml. per hour. The current used did not exceed 10 ma., and when the effects of rate of flow, width of the wick, and addition of PVP were determined a current of 6 to 7 ma. was employed. This eliminated occasional over-distillation.

For long runs, required for the separation of larger quantities, a buffer of higher ionic strength (0.1) proved to be the most useful. In our experiments cooling was not used and all the experiments were carried out at room temperature during the summer (1952). It is probable that the separations would be facilitated if the experiments had been conducted at lower temperatures. Repeated short runs are to be preferred to longer ones since diffusion takes place, especially at the lower end of the paper curtain and overlapping of components results. When this report was being prepared for publication a recent important contribution to this type of electrophoresis was made by Grassmann and Hannig (8). They were able to separate and collect serum proteins, peptides, and products of collagen when treated with crystalline trypsin. They also discuss the factors involved when the electrodes are arranged along the whole length of the filter paper curtain.

Conclusion

The apparatus described has proved in our hands to be useful in further purification of diphtheria toxoid. It is simple and cheap to construct, easily maintained and flexible in operation. These advantages suggest its wide applicability.

Part II. Separation, Collection, and Evaluation of Components

In this laboratory, purified diphtheria toxoid was subjected to filter paper electrophoresis, and separated into three components by the method described by Poulik (17). Flocculation tests, performed in an agar layer, revealed the flocculating activity to be confined to one of the three components. In order to obtain sufficient amounts of the separated components for quantitative determinations the apparatus described in Part I was constructed.

Experimental

Materials

Toxin was prepared in hog stomach autolysate broth (21). The potency of toxin was 100 Lf. After conversion into toxoid (Lot 0153), the material was ultrafiltered, concentrated, and purified by a recent variant (12) of the method of Moloney and Orr (14). It contained 2500 Lf, 0.8 gamma N/Lf. Before use, to each milliliter of toxoid was added 50 mgm. of PVP.

Methods

Visual Examination of Components

As pointed out in the previous paper, the separated components differ in color. Flocculating activity is confined to the pinkish-brown fraction; the yellow-colored fractions give no flocculation reaction. This color difference served to locate the components. By viewing the dried filter paper curtain through a violet filter, visual examination of the color bands was enhanced, as the nonflocculating component showed a distinct absorption of the violet light.

Flocculation Studies of the Components

(1) A modification of the method of Ouchterlony (16) for the diffusion and flocculation of antigens and antibodies in gels was developed for this purpose. Fifteen milliliters of warm agar (1.5%) are pipetted into a Petri dish and allowed to gel. Two rows of penicillin cups are pressed gently into the agar so that they are opposite one another in pairs, separated by a measured distance of 1 cm. One-tenth milliliter of antitoxin (crude immune horse serum containing 600 Lf) is introduced into each cup of one row. Into each cup of the opposite row is pipetted 0.1 ml. of the material separated in the continuous apparatus and contained in the collection flasks. The Petri dishes are placed in the incubator at a temperature of 35° C. After a period of 30 hr., lines of flocculation are only visible in the agar between the antitoxin-containing cups and those cups containing the fraction with flocculating activity. A similar method was successfully used to determine the suitability of different antitoxins for the *in vitro* test of the toxigenicity of *C. diphtheriae* (18).

(2) Ramon Flocculation Test: The well established method was employed. Rabbit Skin Test: The intracutaneous test of Fraser (5, 6) recently modified and described by Greenberg and Gibbard (10) was closely followed.

Results

Of the 56 experiments made, two typical studies will be described. Experiment 45 will be discussed in detail and experiment 50 only briefly because of the shortage of the original material.

Experiment No. 45 (Fig. 3)

Experimental conditions: Room temperature; veronal buffer of ionic strength 0.1 and pH 8.55; current 7 ma.; voltage 350; time 32½ hr.; amount of toxoid used 0.92 ml.; average volume of collected buffer and material, 3.7 ml. per flask.

Visual examination: A yellow-colored band is spread from drip-points No. 2 to No. 5; a pinkish-brown band stretches from drip-points No. 6 to No. 10. This color difference was also evident in the solution in the collection flasks.

Flocculation studies: The Petri dish flocculation method showed the presence of flocculating components in tubes 5, 6, 7, 8, and 9. The remaining nine tubes of the series of 14 showed no trace of a flocculation line. It was assumed that this batch of toxoid was separated into flocculating and non-flocculating components.

The Ramon flocculation test showed 80 Lf/ml. in tube 8; tubes 6, 7, and 9, which were pooled for ultracentrifuge studies, contained 90 Lf/ml. The material flocculated with unusual rapidity.

Animal test: The test was performed with materials collected in tubes 3 and 8. These were selected on the basis of being sufficiently far apart to exclude the likelihood of admixture of the two visible components, difference in color, and in absorption of ultraviolet light as determined by using Beckmann spectrophotometer, model DU. The material in tube 8 was found antigenic, the value being 80–125 Lf/ml. The material in tube 3 showed no antigenic value.

Experiment No. 50 (Fig. 4)

Experimental conditions: Michaelis buffer, pH 6.10; current 8 ma. at 250 volts; room temperature; time 18 hr.; amount of toxoid, 0.5 ml.; average volume of collected material and buffer, 1.35 ml. per flask.

Visual examination: A component of pinkish-brown color stretching from drip-points 2 to 5, one of yellow color situated between drip-points 7 and 8, and a second yellow component spread between drip-points 10 and 11.

Flocculation studies: The Petri dish method showed the presence of flocculating activity in tubes 1, 2, 3, 4, and 5, the remainder showed no lines of flocculation. It was concluded that this batch of toxoid was separated into one flocculating and two nonflocculating fractions.

By determining the flocculating unitage of each tube and estimating the amount of material (approximately 0.15 ml.) purposely left on the paper for visual examination and permanent record, the recovery of the active or flocculating material was calculated. On this bases the recovery was between 70–80%.

Discussion

The main value of the method lies in the fact that diphtheria toxoid of high purity can be further purified with relative ease and with a 70–80% yield. It is not suggested that toxoid per se was separated into fractions though there are indications from experiments with simple electrophoresis that in the ultrafiltered material possibly two flocculating components may be present. The present paper deals only with preliminary results.

The separation of the colored components presented difficulties because of the small difference in their mobilities. In the simple type of filter paper electrophoresis (17), this was not such a difficult problem as in continuous electrophoresis. By using a buffer pH 6.1, close to the isoelectric point of the toxoid, this difficulty was overcome. An attempt was made to correlate

the binding power or toxoid for a dye (azocarmine B) with the amount of flocculating units. Results will be reported later. The flocculating component is stable in the buffer used when stored at 3–5° C., but the nonflocculating components precipitate after a fortnight at the same temperature. The nonflocculating components absorb about 50% more ultraviolet light (at 2800Å) than the flocculating component. Furthermore, they do not stain with azocarmine B and G-X, nor with amidoblack B, thus differing from the characteristic behavior of proteins with respect to these dyes. The ultracentrifuge studies on the separated components are in progress.

Acknowledgments

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THE LIPID COMPOSITION AND WATER CONTENT OF BRAIN, HEART, LUNG, LIVER, GUT, AND SKIN IN THE HOST COMPONENT OF THE ALBINO RAT–WALKER CARCINOMA 256 DUAL ORGANISM[1]

By Eldon M. Boyd, H. D. McEwen, and M. N. Shanas

Abstract

The carcass of albino rats bearing Walker carcinoma 256 contains, at or near death of the dual organism, concentrations of lipids and water altered in the direction of the concentrations of these constituents in the tumor component. The objective of the investigation herein reported was to find the extent to which brain, heart, lung, liver, gut, and skin participated in this shift of lipid and water levels of the host carcass. Total lipid, neutral fat, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, and phospholipid of these tissues were measured by oxidative micromethods in 23 albino rats bearing Walker carcinoma 256 at or near death of the dual organism. The results were expressed as gm. per 100 gm. dry weight of tissue and compared with corresponding values in 23 littermate albino rats without tumors, and differences with a P value of 0.01 or less were noted. Brain could be proved to take no part in the lipid and water shift in the carcass. Heart, lung, liver, and gut participated in the increased water shift. Liver participated in the increased free cholesterol shift. Skin and gut participated markedly in the decreased total lipid, neutral fat, and total fatty acids shift. Gut participated in the increased phospholipid shift. There were no other significant changes in lipid or water levels. These data are further evidence of the metabolism-directing influence of Walker carcinoma 256 on the host.

Introduction

Walker carcinoma 256 is an experimental tumor transplanted in albino rats. Originally an adenocarcinoma discovered on the abdomen of a pregnant albino rat in 1928, and probably of mammary gland origin (8), the histological appearance has now changed to that of a highly anaplastic growth (19). Following inoculation, the tumor grows rapidly and at death of the tumor-host organism some three to four weeks later, the weight of the tumor becomes a considerable proportion of that of the host. The objective of the investigation to be reported below was to determine the influence of the tumor component of this dual organism upon the lipid composition and water content of brain, heart, lung, liver, gut, and skin in the host component.

The tumor component of the albino rat – Walker carcinoma 256 dual organism, like other malignant growths (21, 23), contains a high percentage content of phospholipid, free cholesterol, and esterified cholesterol (10). These high lipid levels are found in the earliest analyzable tumor and maintained at constant values throughout the four weeks of the tumor's growth (6). The neutral fat values are high in the one-week-old tumor but rapidly decline in the second and subsequent weeks (6). The water content of Walker carcinoma 256 averages 85.1% throughout its growth (6).

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In a previous communication to this Journal (5), the homogenized, ground carcass of the host component, after 28 days of tumor growth, was reported to have significantly less neutral fat and significantly more phospholipid, free cholesterol, and water than was found in the carcass of littermate control albino rats bearing no tumor. Each of these shifts in concentration was toward the level of the respective constituent in the tumor component, so that the composition of the host component became more like that of the tumor component. Haven, Bloor, and Randall (12) had previously found that the fatty acids of carcass in the host component become qualitatively (solid/liquid ratio) similar to those of the tumor component as the tumor increases in size. Haven, Bloor, and Dawson (11) later demonstrated that the concentration of lipid phosphorus was high in rats bearing Walker carcinoma 256, the level being similar to the high lipid phosphorus of carcass in young rats, rather than to the lower level found in adult rats without tumors. These data suggest that the tumor has a metabolism directing influence upon the host (5).

Analyzing further the lipid composition and water content of tissues in the host component, Boyd, Connell, and McEwen (5) found that changes in skeletal muscle paralleled those in carcass. In testicle, there was a fall in the concentration of neutral fat, free cholesterol, ester cholesterol, and water toward the corresponding levels in the tumor and no change in the concentration of phospholipid, which was similar in testicles of rats with or without tumors to the concentration in the tumor. In the investigation herein reported, this analysis of changes in lipid composition and water content of the host component has been carried further by including a study of the tissues and organs noted above.

Method

A description of the technique of inoculation of Walker carcinoma 256 and of its growth in albino rats has been previously published in this Journal (6). Factors which affect growth of the tumor have been reported by Walpole (20) and by Talalay, Takano, and Huggins (19). The variety of Walker carcinoma 256 employed was one which has been transplanted in the Department of Pharmacology at Queen's University since 1947. The albino rats used were from a colony which has been maintained in the animal quarters of the Department of Pharmacology at Queen's University since 1937. They were fed Purina fox chow checkers and water ad libitum.

Twenty-three young, male albino rats from this colony were inoculated with Walker carcinoma 256. At or near death, three to four weeks later, the animals were sacrificed by fracture of the cervical spinal column. The dual organism was weighed and the tumor dissected free from the host and weighed. The mean $\pm$ standard deviation weight of the tumor component was 60 $\pm$ 24 gm., of the tumor plus host 211 $\pm$ 71 gm., and of the host minus tumor 151 $\pm$ 43 gm. Twenty-three male albino rats, littermates of the above and not bearing tumors, were sacrificed at the same time as their siblings. The average body weight of the littermate controls at this time was 205 $\pm$ 52 gm.

Aliquots of brain (whole, minced), heart, lung, liver (whole, minced), skin (dorsal thoracic), and gut (duodenum with contents washed out) were removed from the host of the tumor-bearing rats and from littermate controls bearing no tumor, weighed, and dried to constant weight in a Fisher forced draft Isotemp oven for the determination of water content. Other aliquots were weighed, minced, ground with cleaned sand, and extracted with alcohol–ether for the determination of lipids by the oxidative microtechniques of Bloor as modified by Boyd (4). Samples of 0.5 to 1 gm. of all tissues except brain were found to contain amounts of lipids which could be determined with maximum efficiency by the system of analysis employed, the amount for brain averaging 0.25 gm. One half of each extract was used for determination of phospholipid and free cholesterol, one tenth to one quarter for total fatty acid plus total cholesterol and one quarter to two fifths for total cholesterol.

Several minor modifications in the technique of analysis (4) were found by trial to improve the procedure. The addition of 3 to 5 ml. of distilled water to alcohol–ether extracts of tissue before saponification prevented destruction of lipids by concentrated alkali at the end of saponification. The phenol red indicator used at the end of saponification occasionally is extracted by petroleum ether. This could be overcome by using the indicator aurin as a saturated solution in 25% sulphuric acid, added drop by drop until there appeared a yellow color (pH 4) and then one drop in excess. In the final oxidation of the total fatty acids and cholesterol, Nicloux reagent was replaced by 98% sulphuric acid which could be added as approximately 5 ml., thus saving time, producing a more clearly demarcated end point in the thiosulphate titration, and not lowering the completeness of oxidation of lipids.

In the analysis of phospholipid and free cholesterol, the addition of 2 ml. of distilled water to the alcohol–ether aliquot before evaporation on a hot plate, produced several seconds of frothing at the end of evaporation. This signalled to the analyst the near completion of evaporation and prevented overheating.

Results

The results obtained upon brain are summarized in Table I. In column 7 are listed differences between means in the tumor-bearing and nontumor-bearing albino rats, calculated as a percentage of the mean in the nontumor-bearing rat, and such entries have been calculated only when the mean difference had a P value of 0.01 or less as determined after Bradford Hill (13). Values for lipids in the brain of nontumor-bearing littermate control albino rats are similar to those reported by Stoesser, Petri, and McQuarrie (18) and others as reviewed by Bloor (3). The presence of a tumor had no effect upon the lipid composition and water content of brain in the host component. In 1931, Yasuda (22) presented data which suggest that the presence of a tumor

TABLE I

THE LIPID COMPOSITION AND WATER CONTENT OF BRAIN IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AND IN LITTERMATE CONTROL ALBINO RATS BEARING NO TUMOR

Analysis*	Number of animals		Calculation	Tumor-bearing rat	Nontumor-bearing rat	Significant % change in tumor-bearing rat
	Tumor	Control				
Total lipid	14	18	Mean	39.69	38.05	0
			Standard deviation	9.34	11.16	
Neutral fat	15	18	Mean	3.97	2.56	0
			Standard deviation	3.99	2.53	
Total fatty acids	19	22	Mean	19.47	16.19	0
			Standard deviation	6.34	6.97	
Total cholesterol	19	22	Mean	7.73	8.67	0
			Standard deviation	2.66	4.23	
Ester cholesterol	17	19	Mean	1.47	3.14	0
			Standard deviation	2.14	3.53	
Free cholesterol	19	21	Mean	6.73	6.08	0
			Standard deviation	2.44	1.86	
Phospholipid	17	21	Mean	25.89	25.02	0
			Standard deviation	5.94	6.37	
Water (%)	23	23	Mean	81.8	81.3	0
			Standard deviation	1.8	1.6	

* *Values for lipids are expressed as grams per 100 grams dry weight of brain.*

did not affect the brain phospholipid content of host mice in his experiments. These results indicate that brain is not influenced by the disturbance of host tissue lipids and water produced by Walker carcinoma 256.

The results obtained upon heart are summarized in Table II. It may be noted that the presence of a tumor did not affect the lipid composition of heart in the host component of the dual organism. Greenstein (9) tabulated a decline in mean fatty acid and cholesterol ester levels in per cent of wet

weight of host heart of hepatomabearing rats. Sherman, Morton, and Mider (16) have reported that the nitrogen content of heart is unaffected by Walker carcinoma 256, except possibly when the tumor is very large. The metabolic influence of the tumor was apparent, however, in the values for heart water which were significantly elevated and toward the high water content of the tumor. The lipid composition of host heart was not unlike that of the tumor (6).

TABLE II

THE LIPID COMPOSITION AND WATER CONTENT OF HEART IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AND IN LITTERMATE CONTROL ALBINO RATS BEARING NO TUMOR

Analysis*	Number of animals		Calculation	Tumor-bearing rat	Nontumor-bearing rat	Significant % change in tumor-bearing rat
	Tumor	Control				
Total lipid	23	22	Mean	16.59	14.76	0
			Standard deviation	3.58	3.23	
Neutral fat	23	22	Mean	4.33	4.02	0
			Standard deviation	2.59	2.77	
Total fatty acids	23	23	Mean	11.56	10.16	0
			Standard deviation	2.76	2.93	
Total cholesterol	23	23	Mean	1.06	0.92	0
			Standard deviation	0.44	0.58	
Ester cholesterol	23	23	Mean	0.22	0.25	0
			Standard deviation	0.41	0.48	
Free cholesterol	23	23	Mean	0.84	0.67	0
			Standard deviation	0.29	0.25	
Phospholipid	23	23	Mean	11.11	9.74	0
			Standard deviation	3.60	2.27	
Water (%)	23	23	Mean	82.1	79.3	+ 3.5
			Standard deviation	1.8	1.9	

* *Values for lipids are expressed as grams per 100 grams dry weight of heart.*

TABLE III

THE LIPID COMPOSITION AND WATER CONTENT OF LUNGS IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AND IN LITTERMATE CONTROL ALBINO RATS BEARING NO TUMOR

Analysis*	Number of animals		Calculation	Tumor-bearing rat	Nontumor-bearing rat	Significant % change in tumor-bearing rat
	Tumor	Control				
Total lipid	20	23	Mean	17.16	16.51	0
			Standard deviation	4.38	5.39	
Neutral fat	20	23	Mean	4.10	4.32	0
			Standard deviation	3.16	4.95	
Total fatty acids	21	23	Mean	11.17	11.04	0
			Standard deviation	3.08	5.02	
Total cholesterol	21	23	Mean	2.48	2.23	0
			Standard deviation	0.85	0.47	
Ester cholesterol	21	23	Mean	0.43	0.42	0
			Standard deviation	0.44	0.47	
Free cholesterol	23	23	Mean	2.07	1.81	0
			Standard deviation	0.67	0.39	
Phospholipid	22	23	Mean	10.06	9.68	0
			Standard deviation	4.04	3.69	
Water (%)	23	23	Mean	83.2	81.0	+ 3.7
			Standard deviation	2.9	2.9	

* *Values for lipids are expressed as grams per 100 grams dry weight of lungs.*

The results obtained upon lung are summarized in Table III. The values in column 6 correspond to those reported by Bloor (3), in so far as comparison may be made. The presence of the tumor had no effect upon the concentrations of lung lipids of the host, which concentrations were similar to those in the tumor (6), but did cause a significant increase in lung water of the host toward the higher level in the tumor (6). Greenstein (9) notes a decline in fatty acids and phospholipid in per cent of wet weight of lung of hepatoma-bearing rats.

The results obtained upon liver are summarized in Table IV and were similar to those upon heart and lung except that a significant increase, toward levels found in tumor (6), occurred in the concentration of host liver free cholesterol. Begg and Dickinson (2) found liver lipid concentration augmented in Sprague-Dawley rats bearing Walker carcinoma 256 when the rats were force-fed a high fat diet. Reporting his results as percentage of wet weight of liver, Greenstein (9) recorded a decrease in fatty acids, cholesterol, and phospholipid in hepatoma-bearing rats. Jones, Chaikoff, and Lawrence (14) reported

TABLE IV

THE LIPID COMPOSITION AND WATER CONTENT OF LIVER IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AND IN LITTERMATE CONTROL ALBINO RATS BEARING NO TUMOR

Analysis*	Number of animals		Calculation	Tumor-bearing rat	Nontumor-bearing rat	Significant % change in tumor-bearing rat
	Tumor	Control				
Total lipid	19	21	Mean	17.55	16.19	0
			Standard deviation	5.29	3.96	
Neutral fat	19	21	Mean	5.08	3.47	0
			Standard deviation	4.08	2.82	
Total fatty acids	23	23	Mean	13.02	10.74	0
			Standard deviation	6.46	2.74	
Total cholesterol	23	23	Mean	1.31	1.21	0
			Standard deviation	0.61	0.93	
Ester cholesterol	23	23	Mean	0.25	0.52	0
			Standard deviation	0.40	0.94	
Free cholesterol	23	23	Mean	1.06	0.69	+ 54
			Standard deviation	0.37	0.24	
Phospholipid	22	23	Mean	12.59	11.37	0
			Standard deviation	4.62	4.24	
Water (%)	23	23	Mean	77.7	73.2	+ 6.1
			Standard deviation	1.6	2.3	

* *Values for lipids are expressed as grams per 100 grams dry weight of liver.*

that the phospholipid content of liver is depressed when large tumors are growing in mice. The increase in liver water of the host, shown in Table IV, was noted previously by McEwen and Haven (15). Begg (1) confirmed in rats bearing Walker carcinoma 256, a previously reported diminution in liver catalase activity. Sherman, Morton, and Mider (16) found host liver nitrogen augmented in rats bearing a small, but not in rats bearing a large, Walker carcinoma 256.

The results obtained upon skin are summarized in Table V. The lipid composition of skin in rats not bearing Walker carcinoma 256 differs from

TABLE V

THE LIPID COMPOSITION AND WATER CONTENT OF SKIN IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AND IN LITTERMATE CONTROL ALBINO RATS BEARING NO TUMOR

Analysis*	Number of animals		Calculation	Tumor-bearing rat	Nontumor-bearing rat	Significant % change in tumor-bearing rat
	Tumor	Control				
Total lipid	18	15	Mean	9.28	23.60	– 60.7
			Standard deviation	6.28	9.62	
Neutral fat	18	15	Mean	5.67	19.60	– 71.1
			Standard deviation	6.00	9.39	
Total fatty acids	21	16	Mean	7.10	21.05	– 69.7
			Standard deviation	5.66	8.78	
Total cholesterol	22	21	Mean	1.34	1.18	0
			Standard deviation	0.94	0.42	
Ester cholesterol	18	20	Mean	0.71	0.50	0
			Standard deviation	0.73	0.46	
Free cholesterol	19	22	Mean	0.61	0.67	0
			Standard deviation	0.39	0.38	
Phospholipid	21	23	Mean	2.29	2.51	0
			Standard deviation	1.30	0.88	
Water (%)	22	23	Mean	69.1	65.5	0
			Standard deviation	6.1	7.0	

Values for lipids are expressed as grams per 100 grams dry weight of skin.

that of tumor (6) in that there is less phospholipid and less cholesterol fractions and more neutral fat, total fatty acids, and total lipid. Skin of rats bearing Walker carcinoma 256 was found to have lost appreciable amounts of neutral fat, total fatty acids, and total lipid, producing concentrations which were more nearly those of the tumor. There were no significant changes in the cholesterol fractions and phospholipid. Costello, Carruthers, Kamen, and Simoes (7) reported that application of carcinogenic methylcholanthrene to the skin of mice, produced a decrease in skin phospholipid. The mean water content of host skin was elevated toward the concentration in the tumor but the increase could not be proved statistically significant owing to a rather high coefficient of variation.

The results obtained upon gut are summarized in Table VI. The effect of the tumor upon rat gut lipid concentrations was similar to that upon skin except that it was possible to demonstrate, in addition, a significant increase in phospholipid concentration to levels actually beyond those of the tumor (6). There was also a significant increase in gut water of the host toward the level in the tumor (6). Gut phospholipid levels of normal rats reported in Table VI were slightly lower than those of cat intestinal mucosa reported by Sinclair (17).

TABLE VI

THE LIPID COMPOSITION AND WATER CONTENT OF GUT IN THE HOST COMPONENT OF THE ALBINO RAT – WALKER CARCINOMA 256 DUAL ORGANISM AND IN LITTERMATE CONTROL ALBINO RATS BEARING NO TUMOR

Analysis*	Number of animals		Calculation	Tumor-bearing rat	Nontumor-bearing rat	Significant % change in tumor-bearing rat
	Tumor	Control				
Total lipid	22	20	Mean	17.64	26.71	− 34
			Standard deviation	5.94	9.02	
Neutral fat	22	20	Mean	4.27	11.78	− 64
			Standard deviation	3.13	6.22	
Total fatty acids	23	21	Mean	11.81	16.48	− 28
			Standard deviation	3.09	6.48	
Total cholesterol	23	23	Mean	1.65	1.70	0
			Standard deviation	0.58	0.69	
Ester cholesterol	21	23	Mean	0.36	0.47	0
			Standard deviation	0.41	0.85	
Free cholesterol	21	23	Mean	1.29	1.24	0
			Standard deviation	0.35	0.50	
Phospholipid	22	22	Mean	11.56	7.92	+ 46
			Standard deviation	6.32	3.05	
Water (%)	23	23	Mean	84.4	81.0	+ 4.2
			Standard deviation	1.6	2.3	

* *Values for lipids are expressed as grams per 100 grams dry weight of gut.*

Discussion

Boyd, Connell, and McEwen (5) reported that the lipid composition and water content of homogenized carcass of rats bearing Walker carcinoma 256 changed, at or near death of the dual organism, to values which approximated the levels previously found by Boyd and McEwen (6) in the terminal tumor component. From evidence reported above or previously (5), certain organs and tissues participating or not participating in this metabolic shift of host carcass lipid and water levels may be delineated. Brain does not participate

TABLE VII

THE LIPID COMPOSITION AND WATER CONTENT OF WALKER CARCINOMA 256 DETERMINED IN 1953 COMPARED WITH THE SAME VALUES DETERMINED IN 1948-1950

Analysis*	Number of animals		Calculation	1948-1950 Tumor	1953 Tumor	Significant % change in 1953 tumor
	1948-1950	1953				
Total lipid	10	14	Mean	14.22	13.49	0
			Standard deviation	2.91	3.42	
Neutral fat	10	14	Mean	2.78	2.96	0
			Standard deviation	2.94	2.59	
Total fatty acids	10	14	Mean	8.77	8.74	0
			Standard deviation	2.24	2.45	
Total cholesterol	10	15	Mean	2.26	2.04	0
			Standard deviation	0.91	1.60	
Ester cholesterol	10	13	Mean	0.92	0.54	0
			Standard deviation	0.72	0.61	
Free cholesterol	10	13	Mean	1.34	1.41	0
			Standard deviation	0.28	0.58	
Phospholipid	10	14	Mean	8.57	8.68	0
			Standard deviation	2.79	3.95	
Water (%)	10	14	Mean	85.1	85.7	0
			Standard deviation	0.2	4.2	

* *Values for lipids are expressed as grams per 100 grams dry weight of tumor.*

in any proved way. Heart, lung, and liver do not participate in the lipid shift, except for free cholesterol whose concentration is increased in liver as in carcass of the host rat. Their lipid composition is similar to that of tumor. Their water content, normally below that of tumor, is raised toward the level in tumor. Testicle also does not participate appreciably in the lipid shift in the carcass except for an increase in its cholesterol fractions. Its lipid composition is similar to that of tumor. Its water content, normally above the tumor level, declines toward the tumor level. Participating in the considerable loss of neutral fat of the carcass, and consequently of total fatty acids and total lipid, are skin, gut, and muscle. Gut and muscle participate also in the increase in phospholipid and water of the carcass. Muscle, like liver and testicle, participates in the increase in carcass free cholesterol.

All of the organs and tissues examined, except brain, participate, in one or more ways, in the shift of lipids and water levels of the host carcass toward the concentrations in tumor. The concentrations of lipids and water in Walker carcinoma 256 were determined between 1948 and 1950 and reported in 1952 (6). It was deemed advisable to check these values as of 1953 and the results, summarized in Table VII, indicated that no change had occurred in the intervening years.

These results reflect the considerable metabolic effect of Walker carcinoma 256 upon the host albino rat. The general topic of host–tumor biochemical relations has been reviewed by Greenstein (9). Data obtained in this laboratory at Queen's University indicate that all host tissues examined, except brain, participate in the water shift. Brain, heart, lung, liver, and testicle participate to no or a limited extent in the lipid shift. Muscle, skin, gut, and residual carcass participate to a considerable extent in the lipid shift. Practically all shifts in lipid and water levels of host tissues so far examined have been toward the concentration in the tumor. These results suggest further a metabolism-directing influence of the tumor upon the host in the albino rat – Walker carcinoma 256 dual organism.

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THE ANTIBACTERIAL SPECTRUM OF USTILAGIC ACID[1]

By R. W. Reed[2] and M. A. Holder[3]

Abstract

Ustilagic acid is shown to be relatively inactive against common Gram-positive and Gram-negative pathogenic bacteria and against *Mycobacterium tuberculosis*. Serum and urine levels in rabbits following oral administration are much lower than the concentration required for *in vitro* inhibition of most bacteria tested. Human serum depresses the antibacterial effect of ustilagic acid *in vitro*. The drug had no effect on the course of experimental infection in mice.

Introduction

There has been a disturbing increase over the past few years in the number of strains of pathogenic bacteria, particularly staphylococci, resistant to one or more of the currently used antibiotics (1). A continuing search for new and active antimicrobial agents is therefore necessary. Haskins (2) in 1950, while examining strains of the corn smut *Ustilago zeae* observed marked inhibition of some contaminating microorganisms. Further investigation showed the presence of two comparatively stable, fat-soluble, antibiotic substances, one mainly active against some Gram-positive bacteria, the other against some Fungi Imperfecti. Lemieux and associates (4) isolated this material in crystalline form as ustilagic acid, and partially characterized it as a stable glucolipid. Thorn and Haskins (7) later studied the factors affecting growth of *Ustilago zeae* and obtained yields of 8 to 15 mgm. of ustilagic acid per ml. which would make large-scale fermentation commercially feasible. Further investigation by Haskins and Thorn (3) indicated that ustilagic acid was particularly active against *Cryptococcus neoformans*, *Candida albicans*, and some *saprophytic fungi*. *In vitro*, antibacterial activity was limited mainly to Gram-positive bacteria of the few strains tested and to *Mycobacterium smegmatis*. Preliminary tests also suggested that ustilagic acid was relatively nontoxic when administered orally or intraperitoneally.

Because of the antibacterial potentialities of this easily produced agent, further study of its antibacterial spectrum was undertaken. This report deals with the results of these studies.

Materials and Methods

Ustilagic acid (UA) is insoluble in water. It was therefore necessary to find a solvent innocuous to bacteria before *in vitro* sensitivity tests were attempted. Lemieux *et al.* (4) and Haskins (2) found the drug to be somewhat soluble in methanol, ethanol, and human serum. These solvents as well as glycerol and Sorenson's buffer (pH 7.6) were tried.

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In vitro sensitivity tests were done by two methods:

(*a*) *Plate tests.*—Decreasing concentrations of UA were incorporated in hot nutrient agar. This was poured into Petri dishes and after hardening, the agar surface was streaked with the organism being tested.

(*b*) *Tube dilution tests.*—Decreasing amounts of an alcohol solution of UA were added to sterile nutrient broth, which was then inoculated with 10^5 bacteria in 0.1 ml. of broth. After 24 hr. incubation, the end point was read as the lowest concentration of UA which completely inhibited growth.

UA in gelatin capsules was fed to normal rabbits of about 3 kgm. weight. Blood and urine were collected at appropriate intervals and tested for content of UA. Serial dilutions of serum or urine in broth were inoculated with a test organism of known sensitivity and the end point determined after 24 hr. incubation. For *in vivo* testing of UA, the drug was administered to mice intraperitoneally, dissolved in human serum at appropriate concentrations.

Experimental and Results

Solubility of Ustilagic Acid

Various solvents innocuous to bacteria or which would be readily volatilized were tried to determine the most satisfactory vehicle for *in vitro* sensitivity testing. Results of these trials are shown in Table I. Ethanol 70% and methanol 70% were equally good, while human serum was poorest in terms of μgm./ml. dissolved. Ethanol 70% was chosen as the solvent for UA.

TABLE I

SOLUBILITY OF USTILAGIC ACID

Solvent	Conc. UA, μgm./ml.		Temp.	Time	Comment
	Maximum	Optimum			
Ethyl alcohol 60 to 80%	10,000	5000	22° C.	15 min.	Less time if shaken
Methyl alcohol 70 to 100%	10,000	5000	22° C.	20 min.	
Glycerol	5000	2500	90° C.	30 min.	
Sorenson's buffer pH 7.6	4000	1500	90–100° C.	15 min.	
Human serum	2500	1000	37° C.	12 hr.	One hour, if shaken continuously

Effect of Solvents on Antibiotic Activity

Tube dilution tests using UA dissolved in alcohol were run in parallel with agar plate tests. End points were the same by both methods with all bacteria so tested. The final concentration of 2.8% alcohol did not appear to decrease the activity of UA, nor to effect growth as compared with that in alcohol-free

controls. Using alcohol solutions of UA as controls, the effect of other solvents on *in vitro* sensitivity was determined. The results of such tests using *Bacillus cereus* and *Bacillus megaterium* are shown in Table II.

TABLE II

EFFECT OF SOLVENTS ON ACTIVITY OF UA AGAINST *B. cereus* AND *B. megaterium*

	Inhibiting conc. of UA in μgm./ml.						
	400	100	25	6.25	1.56	0.39	Control
Ethanol 70%	−	−	+	+	+	+	+
Human serum	+	+	+	+	+	+	+
Sorenson's buffer pH 7.6	+	+	+	+	+	+	+
Glycerol 1%	−	−	+	+	+	+	+
8%	−	−	−	+	+	+	+
10%	−	−	+	+	+	+	+

\+ = *Growth.*
− = *No growth.*

The end points on the two species shown were identical in all cases. Human serum and Sorenson's buffer pH 7.6 appeared to cause an eightfold decrease in activity. A final concentration of 1% or 10% glycerol in the test medium showed the same effect as 70% ethanol. However 8% glycerol appeared to potentiate the action of UA not only against these two species, but also against two strains of *Proteus mirabilis*, *Bacillus subtilis*, and *Bacillus brevis*. This effect has not been investigated further.

Effect of Storage on Antibiotic Activity

It was felt desirable to determine the effect of storage on the antibacterial action of UA in both dilute and concentrated solutions. Tube dilution sets were made up in nutrient broth from alcoholic solutions of UA to contain decreasing concentrations of the drug. Duplicate sets were tested immediately using a strain of *Proteus vulgaris* sensitive to between 67 and 70 μgm./ml. of UA. The same organism was used for testing duplicate sets after storage for four and eight weeks at 22° C., 4° C., and − 40° C. As shown in Table III, growth occurred in all tubes regardless of time or temperature of storage. An alcoholic solution of 10,000 μgm./ml., however, showed no loss of potency after four weeks at 4° C.

In Vitro Sensitivity Tests

The tube dilution method was used for *in vitro* sensitivity tests with concentrations of UA from 400 μgm./ml. to 0.39 μgm./ml. by serial 1 : 4 dilutions. Nutrient broth was the usual test medium but for more fastidious

TABLE III

EFFECT OF STORAGE ON DILUTE SOLUTIONS OF UA. TEST ORGANISM: *Proteus vulgaris*

Storage conditions		Inhibiting conc. of UA in μgm./ml.			
Temp.	Time	100	85	70	Control
Freshly prepared		−	−	−	+
22° C.	4 wk.	+	+	+	+
	8 wk.	+	+	+	+
4° C.	4 wk.	+	+	+	+
	8 wk.	+	+	+	+
− 40° C.	4 wk.	+	+	+	+
	8 wk.	+	+	+	+

\+ = *Growth.*
− = *No growth.*

organisms, either brain heart infusion broth or fluid thioglycollate medium was used. An alcohol solution of UA was used to prepare the test series in all cases. Three hundred stock and freshly isolated pathogenic and saprophytic bacteria have been tested, and the results are shown in Table IV.

Only 10 of 300 strains tested were inhibited by 25 μgm./ml. or less of UA; 37.5% were not inhibited by 400 μgm./ml.; 35% were inhibited by 400 but not by 100 μgm./ml.; and 23% by 100 but not by 25 μgm./ml. UA is equally ineffective against both Gram-negative and Gram-positive organisms. *Micrococcus pyogenes*, which is becoming a treatment problem owing to hospital infections with strains resistant to several antibiotics (1), requires very high concentrations of UA for inhibition. The majority of strains tested were freshly isolated from hospital infections. Strains of *Proteus* and *Pseudomonas* were not inhibited.

Haskins (2) found UA to be active against *Mycobacterium smegmatis*, and speculated on its possible activity against *M. tuberculosis*. A number of freshly isolated strains of *M. tuberculosis* were tested in the hope that some effect would be manifested by UA. These tests were done in Dubos's medium and also by a slide culture method currently being studied in this laboratory (5) by which both growth of tubercle bacilli and antibiotic sensitivity tests (6) can be observed in one to two weeks instead of the usual three to eight weeks. Results by the two methods of testing were similar but UA proved to be inactive against *M. tuberculosis*.

Treatment of Experimental Infections

Fifty mice each weighing 18 to 23 gm. were injected intraperitoneally with 0.1 ml. of 24 hr. broth culture of *Streptococcus pyogenes* (Strain H8). This strain was inhibited *in vitro* by 400, but not by 100 μgm./ml. of UA. One group of 20 mice were immediately given 2500 μgm. of UA in human serum

TABLE IV

In vitro SENSITIVITY OF BACTERIA TO UA

Organism	Strains tested	Strains inhibited by UA in μgm./ml.						
		>400	400	100	25	6.25	1.56	0.39
M. pyogenes	109	11	62	36	—	—	—	—
S. lutea	1	—	—	—	1	—	—	—
S. pyogenes	13	8	4	1	—	—	—	—
S. viridans	4	4	—	—	—	—	—	—
S. faecalis	12	10	2	—	—	—	—	—
D. pneumoniae	3	2	1	—	—	—	—	—
N. catarrhalis	4	1	1	2	—	—	—	
C. diphtheriae	4	1	3	—	—	—	—	—
Diphtheroids	5	1	2	2	—	—	—	—
Bacillus species	26	2	7	11	6	—	—	—
Clostridium species	4	2	2	—	—	—	—	—
M. tuberculosis var. *hominis*	12	—	12	—	—	—	—	—
M. tuberculosis var. *avium*	2	—	—	2	—	—	—	—
E. coli	22	19	2	1	—	—	—	—
A. aerogenes	7	6	—	1	—	—	—	—
K. pneumoniae	3	3	—	—	—	—	—	—
Paracolobactrum species	4	2	2	—	—	—	—	—
Proteus species	29	10	2	14	—	—	—	3
P. aeruginosa	18	18	—	—	—	—	—	—
S. typhosa	2	1	—	1	—	—	—	—
S. typhimurium	2	2	—	—	—	—	—	—
S. schottmeulleri	2	2	—	—	—	—	—	—
S. enteritidis	1	1	—	—	—	—	—	—
S. dysenteriae	1	—	1	—	—	—	—	—
S. paradysenteriae	1	1	—	—	—	—	—	—
S. alkalescens	1	1	—	—	—	—	—	—
B. anitratum	1	1	—	—	—	—	—	—
A. faecalis	2	2	—	—	—	—	—	—
Achromobacter species	2	1	—	1	—	—	—	—
S. marcescens	3	1	2	—	—	—	—	—
Total strains	300	113	105	72	7	0	0	3

by the same route while a second group of 20 received 1000 μgm. of UA. These amounts in 20 gm. mice are equivalent to 125 and 50 μgm./kgm. of body weight respectively. The remaining 10 mice served as untreated controls. The death rate in both treated groups was 90% in 72 hr., while that in the untreated control group was 70%. It is apparent that UA had no effect on the course of this streptococcal infection in mice. Ninety-six per cent of bacterial strains tested required 100 μgm./ml. or more for *in vitro* inhibition, so it was not considered worthwhile to do further *in vivo* experiments

Serum and Urine Levels of UA

In view of the high *in vitro* concentrations required for inhibition, serum and urine levels have been determined following single and multiple oral doses of UA. The test organism used was a strain of *Bacillus cereus* var. *mycoides* which was inhibited *in vitro* by 17.5 μgm./ml. Samples of urine or serum were serially diluted in nutrient broth. The lowest concentration which

could be detected was 35 μgm./ml. of serum or urine. As shown in Table V, doses of 200 mgm./kgm./day whether given at one time or in divided doses, failed to produce serum levels as high as 35 μgm./ml. A single 200 mgm./kgm. dose gave a urine level of 140 μgm./ml. in 4 hr. but this fell off very rapidly to 35 μgm. at 12 hr. Consideration of the *in vitro* concentrations required for inhibition of 96% of tested strains makes it apparent that UA has no place in systemic treatment of common bacterial infections. A dose of 200 mgm./kgm./day amounts to 15 gm./day for an average 150 lb. man, which is impracticable and probably approaches the toxic dose.

TABLE V

SERUM AND URINE LEVELS OF UA IN RABBITS* FOLLOWING ORAL ADMINISTRATION

Dosage	Hours after first dose	Conc. of UA in μgm./ml.	
		Serum	Urine
200 mgm./kgm./day in one dose	4	< 35	140
	8	< 35	—
	12	< 35	< 35
	24	< 35	70
200 mgm./kgm./day in divided doses q6h	4	< 35	70
	8	< 35	—
	12	< 35	< 35
	24	< 35	< 35

* *Two rabbits were used in each case.*

Discussion

Early reports on the antibacterial activity (2) of ustilagic acid (Ustizeain B) offered some suggestion that this drug might prove useful in the treatment of certain human infectious processes. Large-scale production of UA is relatively simple and yields as high as 20 mgm./ml. of culture medium have been reported (7). Further investigation of the antibacterial spectrum of ustilagic acid was therefore considered worthwhile.

It is generally agreed that for an antibiotic to be effective against an organism *in vivo*, serum levels at least equal to or higher than the *in vitro* sensitivity of that organism must be attained. Such levels are not possible following oral administration of UA. Rabbits fed 200 mgm./kgm./day (equivalent to 15 gm./day in man) consistently showed serum levels of less than 35 μgm./ml. At the same time, 96% of 300 common pathogenic and saprophytic bacteria required 100 μgm./ml. or more for inhibition *in vitro*. It has been demonstrated also that human serum decreases the antibacterial activity of UA from four to eight times. Mice infected with *Streptococcus pyogenes* showed no response to treatment with 125 mgm./kgm. of UA.

It is apparent from the above findings that ustilagic acid has no place in the systemic treatment of bacterial infections in man. It is doubtful too, whether topical use of UA would be effective in treating superficial infections.

Acknowledgments

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INDUCED VARIATION AND STRAIN SELECTION OF *PENICILLIUM CHRYSOGENUM* IN RELATION TO TITER OF NATURAL PENICILLINS[1]

By Leone Farrell

Abstract

Penicillium chrysogenum strain Wisconsin Q176 was used as the parent for a sequence of six experiments in combined irradiation and selection of strains yielding high titers of penicillin with N-(2-hydroxyethyl)-phenylacetamide as the precursor for benzylpenicillin. Altogether, 124 colonies were tested. The six strains chosen as best in their respective experiments were compared. Visible changes were found in color and amount of sporulation and in loss of the deep yellow pigment characteristic of fermentation with Q176. Under standard conditions of test, the peak titer of total penicillin increased in a stepwise fashion from 1285 units/ml. to 2575 units/ml. With appropriate medium, the best strain produced nearly 3000 units/ml., of which more than 95% was benzylpenicillin. In the absence of added precursor, the percentage of *p*-hydroxybenzylpenicillin produced by new isolates increased from 13% of 600 units/ml. to more than 30% of 1000 units/ml. The amount of penicillin F was quite constant, while that of other types was not changed with any apparent regularity. The change in reaction during rapid production of penicillin differed in the degree of acidity noted. When other common precursors for benzylpenicillin were used, about 50% more total penicillin was found than in the control without precursor. The total titer was never as high as with suitable amounts of the particular precursor which had been used in isolating the strains. With each irradiation experiment, the strain deemed best showed increased ability to convert the chosen precursor to benzylpenicillin.

Introduction

Since Demerec (6) successfully applied the technique of irradiation to *Penicillium chrysogenum* to increase the yield of penicillin, the use of radiations has been extended to various cultures which produce antibiotics. The review by Raper (11) gives many references. Remarkable in its results, most of this work is also remarkable for the enormous number of strains isolated and tested. On the whole, screening tests carried out under conditions differing widely from those intended for final use have been disappointing. When a program of irradiation was begun in these Laboratories early in 1949, it was evident that the number of assays which could be assigned to it was limited, and the decision was taken to rely upon a sequence of treatment combined with selection of a few strains rather than upon wide testing after single irradiation treatments. The present report traces the course of development in a single line from *P. chrysogenum* strain Wisconsin Q176 (2) of a series of strains which produce up to three times the titer of penicillin produced by the parent under the same conditions, and 50% more than the highest noted for Q176 (5), and shows several differences observed in these strains in comparison with each other and with Q176.

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Materials and Methods

Culture

Spores from a first transfer of *P. chrysogenum* strain Wisconsin Q176 were dried *in vacuo* from the frozen state in a mixture of 50% sand and potting soil and stored in the refrigerator. Under the experimental conditions, the parent culture in a series of 10 consecutive trials gave a peak titer of penicillin of 970 units/ml., with confidence limits at the 5% level of 955 to 985 units/ml. Rapid mass transfer of this strain on corn steep agar gave a culture which produced 1200–1300 units/ml. under the same conditions. A single colony isolate, No. 129, from this stock was used for irradiation. Corn steep agar was used for slopes for all experimental isolates.

Irradiation

Suspensions of predominantly single spores were prepared in an accepted way (12). Early tests showed that aerosol O.T. 0.01% reduced the viability in 30 min., but not detectably in 10. Dilutions were therefore made out of the wetting agent within 10 min. in every instance. Decimal dilutions were made in sterile water and 0.1 ml. spread on potato dextrose agar plates. Microscopic observation after a few hours' incubation indicated that the spores were well separated by this technique. A sufficiently wide range of dilutions ensured that plates with not more than a dozen colonies would be available for isolation when at least 98% of the spores were killed. Plates were exposed 18 cm. below a C.G.E. 30 watt germicidal lamp for 75, 90, and 120 sec. They were incubated at 26° C. for four to six days, the resulting colonies counted on control and irradiated plates, and those with not more than 2% survival chosen for isolation. As far as possible, spores were transferred from every colony on any plate which was used at all. In the first four experiments, spores were removed for test as soon as the slopes were well covered, usually in four or five days, but seven strains were found which failed to sporulate in three weeks. No attempt was made to test for production of penicillin with these seven. In later experiments, only slopes which sporulated within a week were used.

Corn steep agar.–corn steep solids, 3%; lactose, 3.5%; glucose, 0.5%; zinc sulphate heptahydrate, 0.0044%; adjusted to pH 5.3; calcium carbonate, 1.0%; agar, 2.0%. It is important that the solid material be in suspension when the slopes solidify.

Sporulation broth (Foster *et al.* (7)).–containing 0.05% N-(2-hydroxyethyl)-phenylacetamide (3). Liter flasks containing 180 ml. broth were inoculated with 1 ml. spore suspension from slopes. Test indicated that the number of spores used was not significant except in time of sporulation.

Fermentation broth (Adapted from Moyer and Coghill (10)).–lactose, 4%; corn steep solids, 3.5%; cerelose, 1.5%; zinc sulphate heptahydrate, 0.0044%; calcium carbonate, 1%; N-(2-hydroxyethyl)-phenylacetamide, 0.05%. Liter flasks containing 180 ml. medium with 0.5 ml. soybean oil were inoculated with 4.5 ml. sporulation broth culture.

Incubation and testing.–Flasks were incubated at 24–25° C. on a Gump shaking machine operated at 250 r.p.m. Samples for assay of total penicillin were taken daily from the fourth to the seventh or eighth day. Duplicate flasks were used throughout and assayed separately at three levels by the conventional cup-plate method. For paper-partition chromatography, samples from duplicate flasks were pooled. The percentage composition of the penicillin was calculated by the method of Karnovsky and Johnson (9) for whole strips. The separation of the K constituents was variable. For the tables, all have been added together as penicillin K. Determination of pH was made with a glass electrode. Standard error is used in this report as the measurement of variation.

Results

Titer of Total Penicillin

The results of four successive experiments combining irradiation and selection are presented in Table I.

Table I traces the course of repeated irradiation combined with selection of high-yielding strains, beginning with a single-colony isolate, No. 129, derived from Q176, which gave a peak titer of penicillin of 1200 units/ml. under the experimental conditions; and ending after four repetitions of the combined treatment with the isolation of four strains, out of a total of 94 colonies tested, which gave 1800–1900 units/ml. under the same conditions. As may be seen, the first three experiments gave chiefly poor strains. Work subsequent to any reported here indicates that the large proportion of these is due at least in part to the wetting agent, rather than to irradiation. When strain 13 from experiment C was isolated, it seemed unlikely that the increase to 1335 units/ml. was significant. When it was irradiated, several distinctly improved strains appeared. Study of these showed fundamental changes in metabolism. The routine medium for testing new strains was changed, by increasing the concentration of corn steep solids to 4% in fermentation broth, and that of the precursor for penicillin G to 0.15% in seed and fermentation flasks. Combined irradiation and selection was continued for two more experiments, in which a further 30 colonies were tested without other changes in the routine, and in both, strains were isolated which gave still higher titers.

Comparison of Successively Isolated Variants

No attempt has been made to characterize these strains in detail, but a few differences are obvious. In streak tests on purity-testing media, strains A8 and B14 are indistinguishable from each other and from Q176. In sporulation broth, A8 resembles Q176, but B14 is distinctly slower to form spores and usually gives only slight green coloration in five days. Strain C13 and all subsequent strains give more restricted growth on all solid media tested, with paler spores, and in sporulation broth the color is closer to gray than to green. Plainly with strain D7 and possibly with strain C13, the deep yellow color characteristic of fermentation with Q176 disappeared, and has not been

TABLE I

PENICILLIN PRODUCED IN SHAKEN FLASKS BY PROGENY OF SINGLE COLONIES FROM SERIALLY REPEATED IRRADIATION COMBINED WITH SELECTION OF STRAINS GIVING HIGH TITERS

Ultraviolet irradiation	Strain irradiated	Strain derived from	Max. titer of penicillin (units/ml.) produced by cultures[1]								
			Before irradiation	From single colonies isolated after irradiation (number in group/number tested)							
				0–250	250–500	500–750	750–1000	1000–1250	1250–1500	1500–1750	1750–2000
A	P129	Q176 Without irrad.	1200	1/26	2/26	3/26	10/26	10/26			
B	A8	Irrad. of P129	1210		1/26	3/26	10/26	12/26			
C	B14	Irrad. of A8	1235	1/23	5/23	3/23	4/23	9/23	1/23		
D	C13	Irrad. of B14	1335	1/19	1/19	2/19	1/19	1/19	5/19	4/19	4/19

[1] *Medium (%): lactose, 4; cerelose, 1,5; corn steep solids, 3.5; zinc sulphate heptahydrate, 0.0044; N-(2-hydroxyethyl)-phenylacetamide, 0.05; pH 5.6; calcium carbonate, 1.0.*

observed with later strains. It is uncertain whether these are "pigmentless" strains in the sense of Anderson *et al.* (1), because some experimental conditions have given rise to a little color, particularly when broths become unusually alkaline. Under normal conditions, none is apparent and it has not caused difficulty in purification, at least with the methods used in these laboratories (Moloney and Tosoni, unpublished). Although the strains seem quite stable and have retained their characteristics in at least 12 generations of mass transfer and single colony isolations, the spores from C13 and all later strains have proved rather delicate. Many die during lyophilization, and the survivors die out slowly during storage. None of many methods of preservation which have been tried has been entirely satisfactory.

The six strains chosen as best in their respective experiments were tested simultaneously in the modified control medium for change in pH and titer of total penicillin during fermentation and for composition of the penicillin produced on the sixth day. Data from this experiment are presented in Fig. 1 and Table II.

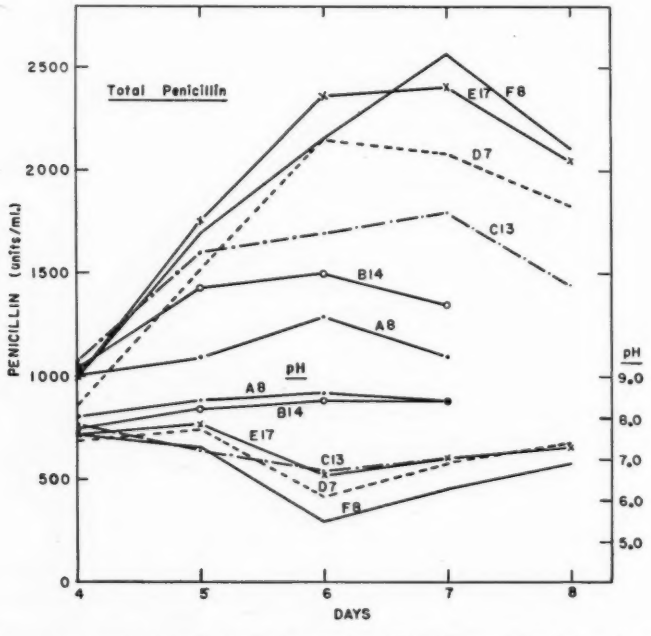

FIG. 1. Total penicillin and change in reaction during fermentation by variant strains in medium containing 0.15% N-(2-hydroxyethyl)-phenylacetamide.
Strain B14 selected after irradiation of strain A8; strain C13 selected after irradiation of strain B14; and so forth.
Composition of medium, expressed in per cent: lactose, 4; cerelose, 1.5; corn steep solids, 4; zinc sulphate heptahydrate, 0.0044; calcium carbonate, 1.0; N-(2-hydroxyethyl)-phenylacetamide, 0.15.

The peak titer of total penicillin increased stepwise with each strain although the difference at each step is not large. As other workers have found (1), there has been a tendency for the level to shift up or down in different tests, but the relative position of strains is usually maintained. Differences in titer found among these strains were also observed when the better ones were used in large fermentors. The curves showing the change in pH during fermentation by strain A8 and by B14 are similar to those found with Q176 and reported by others (13). A distinct change is seen with strain C13, which is marked with D7 and F8. The sharp decrease about the fifth day is characteristic, and notable when it is associated with the large increase in titer of penicillin. No attempt was made to control pH.

TABLE II

TOTAL TITER AND CHROMATOGRAPHIC ANALYSIS OF PENICILLIN PRODUCED ON THE SIXTH DAY BY VARIANT STRAINS

Strain[1]	Total penicillin, units/ml.	Composition, units/ml.				
		X	G	F	Dihydro-F	K
A8	1285	0	1200[2]	—	—	—
B14	1500	0	1400	100	0	0
C13	1690	0	1537	94	40	27
D7	2145	28	1823	98	89	106
E17	2360[3]	41	1985	110	90	131
F8	2145[3]	31	1817	99	108	90

[1] *Strain B14 selected after irradiation of A8, strain C13 selected after irradiation of B14, and so forth. Corn steep medium contained initially 0.15% N-(2-hydroxyethyl)-phenylacetamide.*

[2] *G estimated by comparison of zone diameter with those from the other strips; all other strips calculated independently.*

[3] *Peak titers on seventh day, 2405 and 2575 respectively.*

Table II sets out the results of assay for total penicillin and chromatographic analysis on the sixth day. Using the figure of 5000 units/ml. as the theoretical conversion of 1.5 mgm./ml. of ethanolamide of phenylacetic acid to sodium benzylpenicillin, it may be calculated that the amount of penicillin found on the sixth day represents a gradual increase in coversion from 24% by strain A8 to 35–40% with the last three strains in the table. In other experments under the same conditions strains Q176 and No. 129 produced about 1350 units/ml. of which 60% and 90% respectively was penicillin G, or a conversion of 17% and 25% of the precursor. Table II also shows that all the types of penicillin except F, which remained constant at about 100 units/ml., tended to increase, so that the increase in total penicillin was greater than can be accounted for by increase in benzylpenicillin. This may be interpreted to mean that the acidity of the broths with the later strains is favorable to preservation of penicillin as it is formed. The long continued rise in total penicillin, which usually reaches its peak on the seventh day, as with strain F8 in this test, but often continued till the eighth, would be consistent with this view. Experiments described below indicate that this is not a complete

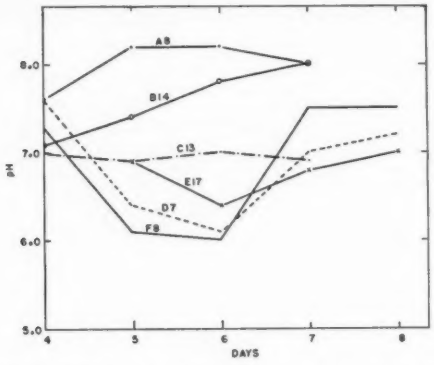

FIG. 2. Change in reaction during fermentation of corn steep medium by variant strains without added precursor.
Medium as in Fig. 1, without precursor.
Strain B14 selected after irradiation of strain A8, strain C13 selected after irradiation of strain B14, and so forth.

explanation however. These strains were also studied in medium to which no precursor was added. For these tests, the inoculum was prepared without precursor.

When strain A8 was cultivated in broth without added precursor, the titer of total penicillin reached its peak, 695 units/ml., on the fifth day and declined slowly thereafter, while the reaction remained strongly alkaline as is shown in Fig. 2. Broth from B14 showed a slower rise in pH and a continued increase in titer until the peak of 820 units/ml. was found on the seventh day. Strain C13, which showed no marked change in reaction after the fourth day, reached its peak titer on the sixth day, and showed no more than experimental variation thereafter. The other three strains showed the same sharp decrease in pH as occurred in the presence of G precursor, the titer reached its peak of about 1000 units/ml. on the fifth or sixth day and did not decline by the eighth. The total penicillin found on the sixth day and its composition are shown in Table III.

TABLE III

TOTAL TITER AND CHROMATOGRAPHIC ANALYSIS OF PENICILLIN PRODUCED BY VARIANT STRAINS ON THE SIXTH DAY IN CORN STEEP MEDIUM WITHOUT ADDED PRECURSOR

Strain	Total penicillin, units/ml.	Composition, units/ml.				
		X	G	F	Dihydro-F	K
A8	590	80	88	206	121	94
B14	785	212	52	193	150	178
C13	910	365	65	202	145	130
D7	895	216	103	216	180	180
E17	1085	295	52	—	—	—
F8	885	372	93	204	102	112

The results of chromatographic separation of the penicillin found on the sixth day with strain A8 grown without added precursor are not very different from those found in a limited number of experiments with Q176 (total penicillin 425 units/ml.), or from those reported by Higuchi and Peterson (8) using a chemical method of assay for types (except X) from Q176. The single-colony isolate 129 which had a titer similar to that of strain A8, also resembled it in composition of penicillin. Beginning with B14, however, the increase in the amount of *p*-hydroxbenzylpenicillin is striking. This culture gave proportionately twice as much penicillin X as the increase in titer would account for, while C13 gave three times as much. Chromatographic separation of the penicillins formed by these early isolates has been done only twice in most instances. Strain F8 has been examined five times under these conditions and the average titer in units/ml. based on the percentage of the total penicillin found on the sixth day is: X, 345 ± 49; G, 96 ± 6; F, 232 ± 13; dihydro-F, 111 ± 12; K, 157 ± 18; total penicillin, 936 ± 18. There is no reason to suppose that results for the other strains are more variable. However, emphasis is placed on the fact that chromatographic separations reported here were carried out on samples taken only on the sixth day from standarized experiments. The amount of penicillin F remained rather close to 200 units/ml. as the total titer varied. In this place, it may be said that ethanolamide of caprylic acid had some effect in increasing total titer and the inhibitory zone corresponding to K in one experiment, but not in another. It should be noted that penicillins F, dihydro-F, and K have not been extracted and identified.

Use of Other Precursors for Benzylpenicillin

Strain F8 was used to compare several precursors for benzylpenicillin with the one employed in isolating all the strains reported here. Sodium phenylacetate was used alone, in various concentrations initially and added at intervals during fermentation, in combination with ethanolamine, and with ethanolamide of acetic acid. Phenylacetamide and β-phenylethylamine have been used at different concentrations initially and at intervals after inoculation. In no instance has the titer of total penicillin been raised to an extent comparable with that found with the precursor which was used in the experiments to select the strain for the next irradiation: that is, with ethanolamide of phenylacetic acid. For example, in an experiment in which the control flasks without added precursor gave a peak titer of 1140 units/ml., initial concentrations of 0.05, 0.1, or 0.15% sodium phenylacetate gave titers of 1420, 1340, and 1475 units/ml. respectively, while phenylacetamide in concentrations of 0.025, 0.05, or 0.1% gave titers of 1600, 1630, and 1705 units/ml. respectively. In the same experiment, 0.15% ethanolamide of phenylacetic acid gave a peak of 2375 units/ml. A survey of all the tests indicates that any of these precursors could be used with this strain to give a high percentage of benzyl penicillin, if added at intervals in appropriate amounts: that is to say, this strain has not lost the ability to convert the usual precursors to penicillin G.

TABLE IV

EFFECT OF SODIUM PHENYLACETATE AND N-(2-HYDROXYETHYL)-PHENYLACETAMIDE ON TOTAL TITER AND COMPOSITION OF PENICILLIN FORMED BY STRAIN F8

Initial precursor		Per cent phenylacetic precursor added at		Penicillin, units/ml.		Composition at 6 days, per cent						% Theoretical[1] conversion to G
Phenyl acetic	%	2 Days	4 Days	Peak	6 Days	X	?	G	F	Dihydro-F	K	
None	0	0	0	945	885	42	0	11	23	12	13	—
Sodium salt	0.11	0	0	1260	1250	11	7	42	14	7	19	13
	0.11	0.11 Salt	0	1345	1200	8	11	47	13	14	7	7
	0.11	0.11 Salt	0.11 Salt	1210	1165	4	7	63	7	6	10	6
Ethanolamide	0.05	0	0	1850	1400	8	0	66	12	6	8	55
	0.05	0.11 Salt	0	1975	1630	3	4	74	7	5	7	21
	0.05	0.11 Salt	0.11 Salt	1895	1510	4	3	69	6	5	6	10
	0.05	0.15 Ethanol	0	2815	1885	0	0	87	5	4	4	24
	0.05	0.15 Ethanol	0.15 Ethanol	2560	2015	0	0	85	5	0	10	15
	0.15	0	0	2375	1950	0	0	94	6	0	0	37

[1] *Theoretical conversion: 1.1 mgm. sodium phenylacetate equivalent to 4130 units sodium penicillin G; 0.5 mgm. ethanolamide of phenylacetic acid equivalent to 1667 units sodium penicillin G.*

The striking point is that in the experiments to date other common precursors have given high percentages of comparatively low totals, while the use of the chosen precursor results in high percentages of G in high total titers. Under suitable conditions, up to 3000/ml. has been obtained of which 96% was penicillin G. The special effect of the chosen precursor is shown by results from a typical experiment in Table IV.

The results presented in Table IV are not intended to suggest that these quantities of precursor or intervals of addition are the best. Brown and Peterson (4) showed that 0.05% phenylacetic acid added nine times at 12 hr. intervals gave superior results with strain Q176. The increased percentages of G found with addition at intervals in this and other experiments indicate that numerous small additions would be better with this strain also. As there has been no indication that they would be as effective in increasing the titer as the chosen precursor when it is used under the best conditions so far worked out, they have not been tried. The use of phenylacetic acid has always been associated with the appearance between the zones for X and G on the chromatogram of an unknown inhibitory agent. This agent, which is indicated in Table IV by a question mark, has been found with preparations of phenylacetic acid from three different makers, and was also observed with strain D7. This precursor has not been used with the other strains in this series.

The difference in the amount of penicillin found with these different precursors cannot be explained simply by reference to the change in pH during fermentation, as might have seemed possible from the earliest experiments described above. When 0.05 or 0.15% ethanolamide of phenylacetic acid, or 0.11% sodium phenylacetate was used initially, the curve for pH was similar to that in fermentation without precursor. Addition of sodium phenylacetate during fermentation to flasks which initially contained 0.05% of ethanolamide of phenylacetic acid did not change the shape of the curve appreciably. As shown in Table IV the peak titer in these groups ranged from 1260 to 2375 units/ml. When sodium phenylacetate was added at intervals to flasks which initially contained sodium phenylacetate, or when the ethanolamide of phenylacetic acid was added at intervals to flasks which initially contained the ethanolamide, all the flasks became alkaline by the fifth day and remained so until the experiment was terminated at the eighth day. In these groups the peak titer ranged from 1210 to 2815 units/ml. The conclusion seems warranted that the real improvement in this strain lies in its ability to make use of the particular precursor which was employed in its isolation.

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CONTENTS OF VOLUME 31

ALEXANDER, H. E.—See Zamenhof, S.

ALLARD, C., DE LAMIRANDE, G., and CANTERO, A.—Mitochondrial population of mammalian cells. III. Number of mitochondria per average cell of rat liver tumor induced by 4-dimethylaminoazobenzene. Significance in the comparative study of the mitochondrial fraction properties of normal tissues and tumors, 103.

ALLEN, T., MACLEOD, A. V., and YOUNG, E. G.—On the nutritional requirements of young children with particular reference to calcification, 447.

ANNAU, E.—See Rice, C. E.

ANNAU, E. and KONST, H.—Enzymatic changes in the liver of mice following feeding of the insecticide aldrin (hexachlorohexahydrodimethanonaphthalene), 146.

BABINEAU, L. M.—See Pagé, E.

BARLOW, J. S. and MANERY, J. F.—A comparison of the method of flame photometry with the chemical methods for the determination of cations in tissues, 326.

BEGG, R. W., DICKINSON, T. E., and MILLAR, J.—The effect of benign and malignant tumors on liver catalase activity, 315.

BEGG, R. W., DICKINSON, T. E., and WHITE, A. V.—The influence of some hormonal, dietary, and tumor factors on liver catalase activity in rats, 307.

BERNARD, R.—See Duchesne, D. D.

BEST, C. H., LUCAS, C. C., PATTERSON, J. M., and RIDOUT, J. H.—Some effects of vitamin B_{12} weanling rats consuming hypolipotropic diets, 135.

BEVERIDGE, J. M. R.—See McLean, J. R.

BIGELOW, W. G.—See McBirnie, J. E.

BOULANGER, P.—See Rice, C. E.

BOYD, C. E.—See Boyd, E. M.

BOYD, E. M.—The lipid composition during storage of human plasma, pooled, irradiated, and dried, 84.

BOYD, E. M. and BOYD, C. E.—Antihistaminics and apomorphine-induced vomiting, 320.

BOYD, E. M., MCEWEN, H. D., and SHANAS, M. N.—The lipid composition and water content of brain, heart, lung, liver, gut, and skin in the host component of the albino rat – Walker carcinoma 256 dual organism, 493.

BURTON, A. C.—See Inch, W. R.

BUTLER, G.—Chemistry of the nucleic acids, 222.

CANTERO, A.—See Allard, C.

CHUBATY, W.—See Lepp, E.

CLARKE, D. W. and MONKHOUSE, F. C.—The combination of heparin and bovine serum albumin, 394.

CLARK, E. M.—See Wood, W.

COLLIER, H. B. (With the technical assistance of S. C. MCRAE)—Enzyme inhibition by derivatives of phenothiazine. VI. Inhibition of glyoxalase activity of human and rabbit erythrocytes, 195.

COLLIP, J. B.—See Derrick, J. B.

DAOUST, R.—See Stevens, C. E.

DENEAU, G. A., WAUD, R. A., and GOWDEY, C. W.—A method for the determination of the effects of drugs on the pain threshold of human subjects, 387.

DERRICK, J. B. and COLLIP, J. B.—Further studies on a pituitary factor with specific influence on metabolism, 117.

DICKINSON, T. E.—See Begg, R. W.

DUCHESNE, D. D. and BERNARD, R.—Further studies of lipotropism in the domestic duck (White Pekin), 474.

DUNCAN, D.—See Franklin, A. E.

DUNCAN, D., FRANKLIN, A. E., WOOD, W., and RHODES, A. J.—Cultivation of poliomyelitis virus in tissue culture. V. Observations on virus propagation in certain animal tissues with a synthetic nutrient medium, 75.

ELWYN, D.—Biogenesis of the nucleic acids, 236.

FARRELL, L.—Induced variation and strain selection of *Penicillium chrysogenum* in relation to titer of natural penicillins, 512.

FERGUSON, J. K. W.—See Prueter, R. D.

FLEMING, J. O.—See Linford, J. H.

FRANKLIN, A. E.—See Duncan, D.

FRANKLIN, A. E., DUNCAN, D., WOOD, W., and RHODES, A. J.—Cultivation of poliomyelitis virus in tissue culture. IV. Further observations on virus propagation in human tissues with a synthetic nutrient medium, 64.

FRASER, M. S.—See Tuba, J.

GLEGG, R. E. and LEBLOND, C. P.—Pressure as a possible cause of dissolution and redeposition of bone and tooth crystals, 202.

GOWDEY, C. W.—See Deneau, G. A.

GRAHAM, A. F.—The viruses as nucleoproteins, 279.

HINTON, N. A., NELLES, J. E., and REED, G. B.—The incidence of *E. coli* group 0111 in sporadic infantile gastroenteritis and the sensitivity to antibiotics of the strains isolated, 431.

HOLDER, M. A.—See Reed, R. W.

HNATKO, S. I.—The isolation of bacteriophages for mycobacteria with reference to phage typing of the genus, 462.

INCH, W. R. and BURTON, A. C.—Variation of the hydrogen ion concentration of healthy intact skin, 127.

JAQUES, L. B.—See Lepp, E.

JESKE, I.—See Tuba, J.

KARACHI, H. H.—See McBirnie, J. E.

KONST, H.—See Annau, E.

KOVACS, E.—Further studies on the neurolytic properties of the spinal fluid in health and disease, 109. Autolysis in normal and pathological cerebrospinal fluids, 358. Nucleases in the cerebrospinal fluid. I. Ribonuclease in normal, neurologically normal, and in pathological CSF's, 437.

DE LAMIRANDE, G.—See Allard, C.

LEBLOND, C. P.—See Glegg, R. E.; Stevens, C. E.

LEIDY, G.—See Zamenhof, S.

LEPP, E., CHUBATY, W., and JAQUES, L. B.—Effect of phenylindanedione and dicumarol in experimental frostbite, 173.

LINFORD, J. H. and FLEMING, J. O.—The absorption of light, in the visible and ultraviolet, by certain steroids in sulphuric acid and ethanol solutions, 182.

LUCAS, C. C.—See Best, C. H.

MCBIRNIE, J. E., PEARSON, F. G., TRUSLER, G. A., KARACHI, H. H., and BIGELOW, W. G.—Physiologic studies of the groundhog (*Marmota monax*), 421.

MCCREERY, P. M.—See Ranta, L. E.

MCEWEN, H. D.—See Boyd, E. M.

MCLEAN, J. R. and BEVERIDGE, J. M. R.—The prevention of liver necrosis by alphatocopherol, methionine, and cysteine by oral and parenteral routes, 417.

MACLEOD, A. V.—See Allen, T.

MADSEN, N. B. and TUBA, J.—The role of alkaline phosphatase in intestinal absorption. I. The kinetics of phosphatase action on various substrates, 1.

MAINLAND, D. and SUTCLIFFE, M. I.—Statistical methods in medical research. II. Sample sizes required in experiments involving all-or-none responses, 406.

MANERY, J. F.—See Barlow, J. S.

MILLAR, J.—See Begg, R. W.

MONKHOUSE, F. C.—See Clarke, D. W.

NELLES, J. E.—See Hinton, N. A.

PAGÉ, E. and BABINEAU, L. M.—The effects of diet and cold on body composition and fat distribution in the white rat, 22.

PATTERSON, J. M.—See Best, C. H.

PEARSON, F. G.—See McBirnie, J. E.

PLUMMER, P. J. G.—See Rice, C. E.

POULIK, M. D.—Purification of diphtheria toxoid by continuous electrophoresis on filter paper, 485.

PRUETER, R. D., WARSON, M. D., and FERGUSON, J. K. W.—Sex difference in resistance of rats to thyroid hormone, 99.

RANTA, L. E. and MCCREERY, P. M.—The antigenicity of cholera vaccine prepared in fluid medium, 338.

RAWLINSON, H. E.—See Tuba, J.

REED, G. B.—See Hinton, N. A.

REED, R. W.—Slide culture of tubercle bacilli. I. Media and methods, 367.

REED, R. W. and HOLDER, M. A.—The antibacterial spectrum of ustilagic acid, 505

RHODES, A. J.—See Duncan, D.; Franklin, A. E.; Wood, W.

RICE, C. E., BOULANGER, P., and PLUMMER, P. J. G.—Parallel studies of complement and blood coagulation. VIII. The effect of choline and methionine on the changes induced in guinea pigs by the injection of carbon tetrachloride, 162.

RICE, C. E., BOULANGER, P., PLUMMER, P. J. G., and ANNAU, E.—Parallel studies of complement and blood coagulation. IX. The effect of methionine, choline, and cystine on the changes induced by ethionine, 343.

RIDOUT, J. H.—See Best, C. H.

SHANAS, M. N.—See Boyd, E. M.

SHAW, L. G.—See Tuba, J.

SHIMADA, F. T.—See Wood, W.

SMITH, D. B.—Some biological effects of ionizing radiations and their effects on desoxyribonucleic acid, 290.

STEVENS, C. E., DAOUST, R., and LEBLOND, C. P.—The desoxyribonucleic acid of interphase and dividing nuclei, 263.

SYMPOSIUM ON NUCLEOPROTEINS, 222 (See papers by Butler, G., Elwyn, D., Zamenhof, S. *et al.*, Stevens, C. E. *et al.*, Graham, A. F., Smith, D. B.); Round Table Discussion, 302.

SUTCLIFFE, M. I.—See Mainland, D.

SZERB, J. C.—The response of circulating eosinophile cells to morphine and related substances, 8.

TRUSLER, G. A.—See McBirnie, J. E.

TUBA, J. Serum alkaline phosphatase levels in mice of the C_{57}, C_3H, and A strains, 41. Liver glucuronidase activity of rats in various experimental states, 18. See Madsen, N. B.

TUBA, J., RAWLINSON, H. E., FRASER, M. S., and JESKE, I.—Hyperplastic nodules in rat mammary glands following the feeding of 4-acetylaminobiphenyl, 95.

TUBA, J., RAWLINSON, H. E., SHAW, L. G., FRASER, M. S., and JESKE, I.—The effect of age, sex, and previous pregnancy on oxygen uptake of mammary tissue of mice of the $C_{57}BL$, A, and C_3H strains, 400.

TUBA, J. and WIBERG, S.—On rat serum amylase. I. Studies in the normal and the alloxan diabetic animal, 377.

WARSON, M. D.—See Prueter, R. D.

WAUD, R. A.—See Deneau, G. A.

WEBBER, R. V.—See Young, E. G.

WHITE, A. V.—See Begg, R. W.

WIBERG, S.—See Tuba, J.

WOOD, W.—See Duncan, D.; Franklin, A. E.

WOOD, W., CLARK, E. M., SHIMADA, F. T., and RHODES, A. J.—Poliomyelitis in Canadian Eskimos. Laboratory studies. IV. Antigenic typing of virus strains in monkeys and in tissue cultures, 207.

WRIGHT, W. D.—See Zbarsky, S. H.

YOUNG, E. G.—See Allen, T.

YOUNG, E. G. and WEBBER, R. V.—On the origin of human plasma proteins: Electrophoretic analyses in selected pathological states, 45.

ZAMENHOF, S., ALEXANDER, H. E., and LEIDY, G.—Biological activity of the nucleic acids, 252.

ZBARSKY, S. H. and WRIGHT, W. D.—The metabolism of C^{14}-urea in the rat, 151.

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Contents

Page

Nucleases in the Cerebrospinal Fluid. I. Ribonuclease in Normal, Neurologically Normal, and in Pathological CSF's—*Ernest Kovacs* - - 437

On the Nutritional Requirements of Young Children with Particular Reference to Calcification—*Thelma Allen, Ada Y. MacLeod, and E. Gordon Young* - 447

The Isolation of Bacteriophages for Mycobacteria with Reference to Phage Typing of the Genus—*Stephen I. Hnatko* - - - - - - - 462

Further Studies of Lipotropism in the Domestic Duck (White Pekin)—*Denyse D. Duchesne and Richard Bernard* - - - - - - - - - 474

Purification of Diphtheria Toxoid by Continuous Electrophoresis on Filter Paper—*M. D. Poulik* - - - - - - - - - - - 485

The Lipid Composition and Water Content of Brain, Heart, Lung, Liver, Gut, and Skin in the Host Component of the Albino Rat – Walker Carcinoma 256 Dual Organism—*Eldon M. Boyd, H. D. McEwen, and M. N. Shanas* 493

The Antibacterial Spectrum of Ustilagic Acid—*R. W. Reed and M. A. Holder* 505

Induced Variation and Strain Selection of *Penicillium chrysogenum* in Relation to Titer of Natural Penicillins—*Leone Farrell* - - - 512

Contents of Volume 31 - - - - - - - - - - - 523